Universitat de Lleida

Implementación de técnicas moleculares para la detección y cuantificación del agente de biocontrol Pantoea agglomerans CPA-2

Lourdes Soto Muñoz

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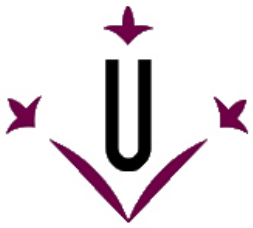

Universitat de Lleida

Escola Tècnica Superior d' Enginyeria Agrària

Departament de Tecnologia d' Aliments

Implementación de técnicas moleculares para la detección y cuantificación del agente de biocontrol *Pantoea agglomerans* CPA-2

Memoria presentada por
Lourdes Soto Muñoz

Para optar el grado de:
Doctorado en Ciencias y Tecnología Agraria y Alimentaria

Directoras de Tesis:
Dra. Neus Teixidó i Espasa
Dra. Rosario Torres Sanchis

Tutora Académica
Dra. Inmaculada Viñas Almenar

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A mi abuela, a mi
mami y a mis hermanas

Tranquila, despacio que llevas prisa
(Ramón Álvar M. Peniche)

Ahorita voy y ahorita vengo…
(Propia)

No encuentro palabras con que expresar mi agradecimiento a todos y cada uno de ustedes que ya sea cerca o en la lejanía, en las buenas y en las malas, sin importar si era de día o de noche, laborable o vacaciones, siempre estuvieron ahí para apoyarme, darme ánimos y fortaleza para continuar en este viaje.

Gracias por creer y confiar en mi, por darme la libertad para partir en busca de este sueño y dejarme escribir mi propia historia, por su paciencia, consejos y enseñanzas.

Gracias por dedicarme su valioso tiempo, tiempo para demostrar su preocupación por mí, tiempo para escuchar mis problemas y ayudarme a buscar solución, tiempo para estar conmigo cuando más lo necesité, tiempo para sonreír.

Gracias, por los momentos que hemos compartido, pero sobre todo, gracias por su amistad, por sus muestras de afecto y cariño, que me alegraron y continuarán alegrándome la vida.

A todos ustedes, MIL GRACIAS.

Los estudios presentados en esta tesis doctoral se han desarrollado en el laboratorio de Patología del programa de Postcosecha del IRTA (Institut de Reserca i Tecnologia Agroalimentàries) de Lleida.
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ÍNDICE

ABREVIATURAS

ACB	Agente de control biológico
UFC	Unidades formadoras de colonias
VBNC	Viable no cultivable
PCR	Reacción en cadena de la Polimerasa
ADN	Ácido desoxirribonucleico
SCARs	Regiones amplificadas caracterizadas y secuenciadas
qPCR	Reacción en cadena de la Polimerasa en tiempo real o Cuantitativa
Cq	Ciclo de umbral
Tm	Temperatura de fusión
MGB	Ligando de unión al surco menor
PMA	Propidio de monoazida

RESUMEN / RESUM / SUMMARY

RESUMEN

El control biológico puede ser una alternativa al uso de fungicidas químicos de síntesis para el control de patógenos. En este sentido, la bacteria *Pantoea agglomerans* CPA-2 es un potencial agente de biocontrol (ACB) eficaz en el control de enfermedades de postcosecha en fruta de pepita y cítricos. No obstante, para implementar y registrar el uso de este ACB como estrategia práctica de control en Europa es necesario realizar una serie de estudios que permitan generar la información requerida por la normativa europea (Nº 546/2011). Algunos de los requerimientos que la normativa establece se refieren a la capacidad del antagonista para colonizar, persistir y propagarse, así como sus posibles rutas de dispersión en condiciones habituales de aplicación. Para obtener dicha información es importante contar con un método de detección que permita identificar y cuantificar al ACB del resto de la microbiota; siendo las técnicas moleculares una de las más adecuadas.

La presente tesis doctoral tuvo como objetivo fundamental el desarrollo de técnicas moleculares para la identificación y cuantificación específica del antagonista *P. agglomerans* CPA-2 en su proceso de formulación y en su entorno de aplicación (pre- y postcosecha). Para cumplir este objetivo, en primer lugar se desarrolló y validó la técnica de qPCR, mediante el diseño de cebadores (SP_2-F/SP_2-R) y una sonda *Taqman*-MGB altamente específicos y sensibles para CPA-2. La técnica de qPCR desarrollada permitió la cuantificación del antagonista inmediatamente después de su aplicación en manzana en condiciones de postcosecha. Sin embargo, sobreestimó la población viable de CPA-2 a medio y largo plazo de conservación debido a que no fue capaz de discriminar entre el ADN de las células viables y las no viables *(Capítulo I)*. Esta limitación se superó con un pre-tratamiento de las muestras antes de la extracción del ADN con el intercalador de ADN, propidio de monoazida (PMA), combinado con la qPCR

(PMA-qPCR) *(Capítulo II)*. Posteriormente, la técnica de PMA-qPCR se aplicó para: *(i)* determinar la supervivencia de CPA-2 después de ser formulada mediante tres técnicas de secado: liofilización, lecho fluido y atomización *(Capítulo III)*; y *(ii)* evaluar la dinámica poblacional del antagonista sobre la superficie de naranjas tratadas en pre- *(Capítulo IV)* y postcosecha *(Capítulo V)*. En todos los estudios, los resultados obtenidos mediante PMA-qPCR fueron comparados con los obtenidos mediante qPCR y el método de recuento en placa. Finalmente, se estudió la persistencia de CPA-2 en el tratamiento de precosecha de cítricos *(Capítulo IV)* y en postcosecha utilizando dos sistemas de aplicación: *drencher (Capítulo I)* en manzanas y línea de confección para cítricos *(Capítulo V)*. La persistencia y presencia del antagonista fue evaluada muestreando las zonas de tratamiento y almacenamiento, así como los equipos de protección de los operarios (EPIs). Las colonias con características fenotípicas similares a *P. agglomerans* fueron confirmadas por PCR convencional.

Los resultados obtenidos de los estudios realizados en esta tesis demuestran la versatilidad de las técnicas moleculares basadas en la PCR (qPCR, PMA-qPCR y PCR convencional) para detectar y cuantificar de *P. agglomerans* CPA-2. En particular, la técnica de PMA-qPCR resultó ser una herramienta útil para detectar y cuantificar las células viables de *P. agglomerans* CPA-2 de forma rápida y específica, así como las células que entran en un estado viable no cultivable (VBCN) como resultado del estrés que sufren durante su formulación y en las condiciones ambientales de aplicación. Además, en esta tesis se demostró la limitada dispersión y baja persistencia de CPA-2 en condiciones habituales de aplicación. Permitiendo de esta manera comprender el comportamiento del antagonista y proporcionando datos valiosos para fines de registro.

RESUM

El control biològic pot ser una alternativa a l'ús de fungicides químics de síntesi per al control de patògens. En aquest sentit, la bactèria *Pantoea agglomerans* CPA-2 és un potencial agent de biocontrol (ACB) eficaç en el control de malalties de postcollita en fruita de llavor i cítrics. No obstant, per implementar i registrar l'ús d'aquest ACB com estratègia pràctica de control a Europa és necessari realitzar una sèrie d'estudis que permetin generar la informació requerida per la normativa europea (Nº 546/2011). Alguns dels requeriments que la normativa estableix es refereixen a la capacitat de l'antagonista per colonitzar, persistir i propagar-se, així com les seves possibles rutes de dispersió en condicions habituals d'aplicació. Per obtenir aquesta informació, és important comptar amb un mètode de detecció que permeti identificar i quantificar l'ACB de la resta de la microbiota; unes de les tècniques més adients serien les moleculars.

La present tesi doctoral va tenir com objectiu fonamental el desenvolupament de tècniques moleculars per a la identificació i quantificació específica de l'antagonista *P. agglomerans* CPA-2 en el seu procés de formulació i en el seu entorn d'aplicació (pre- i postcollita). Per complir aquest objectiu, en primer lloc, es va desenvolupar i validar la tècnica de qPCR, mitjançant el disseny de cebadors (SP_2-F/SP_2-R) i una sonda *Taqman*-MGB altament específics i sensibles per a CPA-2. La tècnica de qPCR desenvolupada va permetre la quantificació de l'antagonista immediatament després de la seva aplicació en poma en condicions de postcollita. No obstant, va sobreestimar la població viable de CPA-2 a mig i llarg termini de conservació degut a què no fou capaç de discriminar entre l'ADN de les cèl·lules viables i les no viables *(Capítol I)*. Aquesta limitació es va superar amb un pretractament de les mostres abans de l'extracció de l'ADN amb l'intercalador d'ADN, propidi de monoacida (PMA), combinat amb la qPCR

(PMA-qPCR) *(Capítol II)*. Posteriorment, la tècnica de PMA-qPCR es va aplicar per a: *(i)* determinar la supervivència de CPA-2 després de ser formulada mitjançant tres tècniques d'assecat; liofilització, llit fluïditzat i atomització *(Capítol III)*; i *(ii)* avaluar la dinàmica poblacional de l'antagonista sobre la superfície de taronges tractades en pre *(Capítol IV)* i postcollita *(Capítol V)*. En tots els estudis, els resultats obtinguts mitjançant PMA-qPCR van ser comparats amb els obtinguts mitjançant qPCR i el mètode de recompte en placa. Finalment, es va estudiar la persistència de CPA-2 en el tractament de precollita de cítrics *(Capítol IV)* i en postcollita, utilitzant dos sistemes d'aplicació: *dréncher (Capítol I)* en pomes i línia de confecció per cítrics *(Capítol V)*. La persistència i presència de l'antagonista fou avaluada mostrejant les zones de tractament i emmagatzematge, així com els equips de protecció dels operaris (EPIs). Les colònies amb característiques fenotípiques similars a *P. agglomerans* van ser confirmades per PCR convencional.

Els resultats obtinguts dels estudis realitzats en aquesta tesi demostren la versatilitat de les tècniques moleculars basades en la PCR (qPCR, PMA-qPCR i PCR convencional) per a detectar i quantificar *P. agglomerans* CPA-2. En particular, la tècnica de PMA-qPCR va resultar ser una eina útil per detectar i quantificar les cèl·lules viables de *P. agglomerans* CPA-2 de forma ràpida i específica, així com les cèl·lules que entren en un estat viable no cultivable (VBCN) com a resultat de l'estrès que sofreixen durant la seva formulació i en les condicions ambientals d'aplicació. A més a més, en aquesta tesi es va demostrar la limitada dispersió i baixa persistència de CPA-2 en condicions habituals d'aplicació. Permetent d'aquesta manera comprendre el comportament de l'antagonista i proporcionant dades valuoses per a temes de registre.

SUMMARY

Biological control can be an alternative to synthetic fungicides in the control of pathogens. In this sense, the bacterium *Pantoea agglomerans* CPA-2 is a potential biocontrol agent effective (ACB) in the control of postharvest diseases in pome fruit and citrus. However, in order to implement and register the use of this ACB as a practical control strategy in Europe it is first necessary to conduct a series of studies to generate the information required by European regulations (No. 546/2011). Some of the requirements established by the regulations refer to the ability of the antagonist to colonize, persist, and spread possible dispersal routes in normal operating conditions. To obtain such information it is important to have a screening method to identify and quantify the ACB of the rest of the microbiota. One of the most appropriate techniques to obtain this data would be a molecular screening method.

The main goal of the present thesis was the development of molecular techniques for the identification and quantification of CPA-2 strain in its formulation and application in the environment (pre- and postharvest). To achieve this objective, the development and validation qPCR technique was first founded by designing primers (SP2-F / SP2-R) and a highly specific and sensitive *Taqman*-MGB probe for CPA-2. The technique developed for the qPCR allowed for immediate quantification of the antagonist after its application in apples during postharvest conditions. However, it overestimated the viable population of CPA-2 in the medium and long term because it was not able to discriminate between the DNA of viable and non-viable cells (*Chapter I*). This limitation was overcome by treating the samples with the DNA intercalator, propidium of monoazide (PMA), combined with qPCR (PMA-qPCR) prior to the extraction of the DNA (*Chapter II*). Futhermore, PMA-qPCR technique was applied to: *(i)* determine the survival of CPA-2

after its formulation by freeze drying, spray drying and fluidized bed drying (Chapter III); and *(ii)* to assess the population dynamics of the antagonist on the surface of pre-treated oranges (*Chapter IV*) and postharvest oranges (*Chapter V*). In all studies, the results obtained by PMA-qPCR were compared with those obtained by qPCR and plate count method. Finally, the persistence of CPA-2 in the treatment of preharvest citrus (*Chapter IV*) and postharvest citrus was studied using two application systems: drenching (*Chapter I*) in apples and packing lines in citrus fruit (*Chapter V*). The persistence and presence of the antagonist was assessed by sampling the processing and storage areas, as well as the protective equipment operators (PPE). Colonies with phenotypic characteristics similar to *P. agglomerans* were confirmed by conventional PCR.

The results presented in this thesis demonstrate the versatility of molecular techniques based on PCR (qPCR, PMA-qPCR and conventional PCR) to detect and quantify *P. agglomerans* CPA-2. In particular, the PMA-qPCR technique proved to be a useful tool to detect and quantify viable cells of *P. agglomerans* CPA-2 in a quick and specific manner, as well as cells that enter a viable but nonculturable state (VBCN) as a result of stress experienced during their formulation and implementation within environmental conditions. In addition, this thesis also displayed limited dispersal and low persistence of CPA-2 in the normal testing conditions in which it was applied and demonstrated. Thus allowing one to understand the behavior of the antagonist and providing valuable data for registration purposes.

INTRODUCCIÓN GENERAL

1. ANTECEDENTES

Las enfermedades en postcosecha provocan importantes pérdidas económicas al sector hortofrutícola (Mari *et al.* 2014). La gran mayoría de los agentes patógenos causantes de enfermedades de postcosecha en cítricos y fruta de pepita son atribuidas a hongos filamentosos, y particularmente, a patógenos de herida estrictos, tales como *Penicillium digitatum* o *Penicillium italicum* causantes de las podredumbres verde y azul en cítricos (Schena *et al.* 2011), y *Penicillium expansum* causante de la podredumbre azul en frutos de pepita (Viñas *et al.* 2013) (Figura 1).

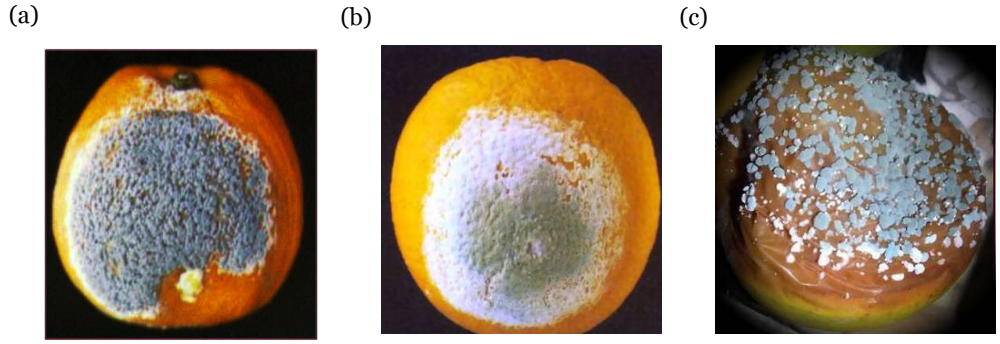


Figura 1. Principales enfermedades de postcosecha en cítricos: (a) Podredumbre verde producida por *Penicillium digitatum* y (b) podredumbre azul por *Penicillium italicum*, y en fruta de pepita: (c) Podredumbre azul causada por *Penicillium expansum*.

La infección del fruto tiene lugar a través de heridas o microheridas producidas en la corteza antes, durante o después de la recolección. En concreto, para que la podredumbre se produzca en los frutos, deben darse las siguientes condiciones: a) nivel de inóculo suficiente en el ambiente; b) contacto entre el inóculo y la superficie de los frutos; c) entrada de la espora en el fruto a través de una herida (incluso no perceptible a simple vista); d) condiciones favorables para que la espora se desarrolle dentro de la

herida; e) susceptibilidad del fruto a la alteración. Por tanto, la incidencia de la podredumbre dependerá de las características intrínsecas del fruto, las condiciones ambientales, las del manejo del fruto durante la recolección y la posterior manipulación en postcosecha (Viñas *et al.* 2013). En conjunto estos factores hacen que la incidencia de las podredumbres sea elevada (normalmente más del 80 % del total de pérdidas por podredumbres) y por tanto, constituyen el eje básico de cualquier estrategia de control.

La forma tradicional de control de estas enfermedades se realiza mediante el empleo de productos químicos de síntesis, por su fácil aplicación, su precio y efectividad. Sin embargo, su uso prolongado y a veces descontrolado ha generado una serie de problemas como son la aparición de cepas de patógenos resistentes a estos productos. A este problema se suma la presencia de residuos en los frutos, con el consecuente incremento de los riesgos para la salud humana y el medio ambiente, así como las crecientes limitaciones que imponen los mercados. Por tanto, existe una clara necesidad de métodos de control que sean nuevas alternativas de control racionales y respetuosas con el medio ambiente.

La necesidad de nuevas estrategias ha originado el desarrollo de una variedad de métodos alternativos a los productos químicos para el control de enfermedades de postcosecha en cítricos (Talibi *et al.* 2014) y en manzana (Moscetti *et al.* 2013). Entre los principales métodos de control encontramos, las prácticas previas a la cosecha incluyendo la aplicación de tratamientos químicos en campo o minimizar las lesiones de frutas, los métodos físicos, químicos y biológicos aplicados en postcosecha. Entre las medidas alternativas para el control de *P. digitatum*, se ha estudiado el uso de la luz UV (Droby *et al.* 1993), tratamientos con calor (tanto aire caliente como la inmersión en agua caliente) (Benyehoshua *et al.* 1987; Plaza *et al.* 2003) y la exposición al ozono (Palo *et al.* 2003) entre otros. De la misma manera, una variedad de medidas alternativas se han sugerido para el control *P. expansum*, entre ellas encontramos: el uso de compuestos químicos de baja toxicidad, como el bicarbonato de sodio y los compuestos naturales (aceites esenciales o polifenoles) (Sanzani *et al.* 2009; Schena *et al.* 2007), baños de agua con calor (Maxin *et al.* 2012) o bien, la combinación de algunas de las estrategias anteriores (D'Aquino *et al.* 2013).

Algunas de estas estrategias se encuentran en fase experimental y resultan menos factibles de utilizar debido a su elevado coste de aplicación y baja efectividad: los fungicidas de bajo riesgo, los tratamientos térmicos (curado) y el control biológico (Lima *et al.* 2006; Palou *et al.* 2003; Torres *et al.* 2007; Usall *et al.* 2001) se han planteado a nivel comercial. Por su parte, el control biológico ha adquirido una gran importancia, como estrategia potencial para el control de enfermedades en postcosecha.

2. EL CONTROL BIOLÓGICO

El control biológico en comparación a los productos químicos de síntesis, presenta claras ventajas dado que los agentes de biocontrol son más seguros, no se acumulan en los alimentos, pueden persistir a lo largo del tiempo, no alteran de forma sustancial el metabolismo del patógeno, y son compatibles con otros sistemas de control. No obstante, su aplicación en postcosecha presenta un gran desafío para el control biológico ya que el ambiente parcialmente controlado, de temperatura y humedad, puede ayudar a alterar el equilibrio en las interacciones entre el huésped y el patógeno a favor del antagonista (Liu *et al.* 2013).

El uso de agentes de control biológico (ACBs) frente a los principales patógenos de cítricos y fruta de pepita se ha convertido en un área de investigación activa. Una extensa lista de antagonistas ha sido descrita por Teixidó *et al.* (2011a) para el control de enfermedades de postcosecha de naranja y manzana, entre los que se incluyen: *Candida oleophila, Pichia anomala* (Lahlali *et al.* 2004), *Bacillus subtilis* (Leelasuphakul *et al.* 2008), *Wickerhamomyces anomalus* (Platania *et al.* 2012) y *Cystofilobasidium infirmominiatum* (Vero *et al.* 2013) para el control de *P. digitatum* en naranja; y *Candida sake* (Viñas *et al.* 1998), *Pantoea ananatis* (Torres *et al.* 2005), *Cryptococcus laurentii* (Li y Tian, 2006), *Metschnikowia fructicola* (Spadaro *et al.* 2013), y más recientemente, *Pichia caribbica* (Cao *et al.* 2013) como efectivos antagonistas de *P. expansum* en manzanas. Como se ha discutido en varias revisiones, la combinación de ACB con otros métodos alternativos de control puede ser un enfoque prometedor para superar algunos inconvenientes de la actividad de los ACBs, y la mejora de su eficacia (Droby *et al.* 2009; Liu *et al.* 2013).

2.1. Desarrollo de un agente de biocontrol en enfermedades de postcosecha

En el momento de seleccionar un microorganismo como ACB en postcosecha, además de estudiar su poder inhibitorio se ha de tener en cuenta muchas características. Según Wilson y Wisniewski (1989) un antagonista ideal de los patógenos de postcosecha ha de ser:

- ✓ Genéticamente estable.
- ✓ Efectivo a bajas concentraciones.
- ✓ Poco exigente en sus requerimientos de nutrientes.
- ✓ Capaz de sobrevivir a condiciones ambientales adversas (incluyendo bajas temperaturas y almacenamiento bajo condiciones controladas).
- ✓ Eficaz contra una amplia gama de patógenos en diferentes tipos de frutas.
- ✓ Capaz de crecer en medios de crecimiento económicos.
- ✓ Poder formularse y conservarse durante largos periodos sin perder capacidad de control.
- ✓ Fácil de aplicar.
- ✓ Compatible con productos químicos utilizados en postcosecha.
- ✓ No perjudicial para la salud humana.
- ✓ Compatible con los procedimientos de procesado comercial.

Adicionalmente, el uso de ACBs para el control de enfermedades de postcosecha de fruta requiere un amplio conocimiento de los mecanismos de acción a través de los cuales estos actúan (Droby *et al.* 2009). A pesar del gran número de investigaciones sobre los microorganismos antagónicos, sus mecanismos de acción no han sido completamente dilucidados para la mayoría de los antagonistas aislados (Janisiewicz y Korsten, 2002). Los mecanismos utilizados por los ACBs suelen ser diversos e incluyen la competencia por nutrientes y espacio, producción de antibióticos e inhibidores de crecimiento de patógenos e inducción de procesos de resistencia secundaria en el huésped, entre otros (Teixidó *et al.* 2011a).

La identificación, desarrollo y comercialización de un producto biológico es un proceso largo y costoso que involucra varios pasos. La figura 2 representa en forma de diagrama de flujo los factores que están involucrados en el desarrollo de un ACB de postcosecha. El desarrollo implica dos fases: el descubrimiento del antagonista y el desarrollo comercial del producto a base del ACB (Nunes, 2012).

Los pasos de la primera fase lo constituyen:

1) El aislamiento y la eficacia evaluada tanto en ensayos de laboratorio como a nivel piloto.
2) La compatibilidad del ACB con diferentes productos y con las prácticas de postcosecha de las centrales hortofrutícolas.
3) El estudio del posible modo de acción del microorganismo antagonista.
4) Los requisitos de crecimiento, que en su momento ayudarán a definir el medio de producción.
5) La mejora de la actividad del ACB.
6) La patente del uso del microorganismo como bioplaguicida.

La segunda fase corresponde al desarrollo comercial del ACB, en ésta se incluyen pasos, tales como:

1) El escalado de la producción a nivel comercial.
2) El desarrollo de un producto formulado.
3) Estudios de mercado, riesgos ambientales y sanitarios.
4) Finalmente, el proceso de registro y otros aspectos regulatorios.

De las dos fases del programa del desarrollo de un ACB, la segunda fase seguramente es la que presenta más desafíos en su realización ya que es necesario el uso de equipos industriales, para la realización del escalado de la producción y/o la formulación, por ejemplo. Además, el tipo de ensayos necesarios para evaluar el riesgo ambiental del antagonista carecen de protocolos definidos para su estudio. Esto sin dejar de considerar que el registro y la reglamentación de ACBs (de la cual hablaremos más adelante) es un proceso largo y costoso. Por tanto, para llevar a cabo estos estudios es frecuente la asociación de los equipos de investigación con una empresa privada que desee comercializar el producto de control biológico.

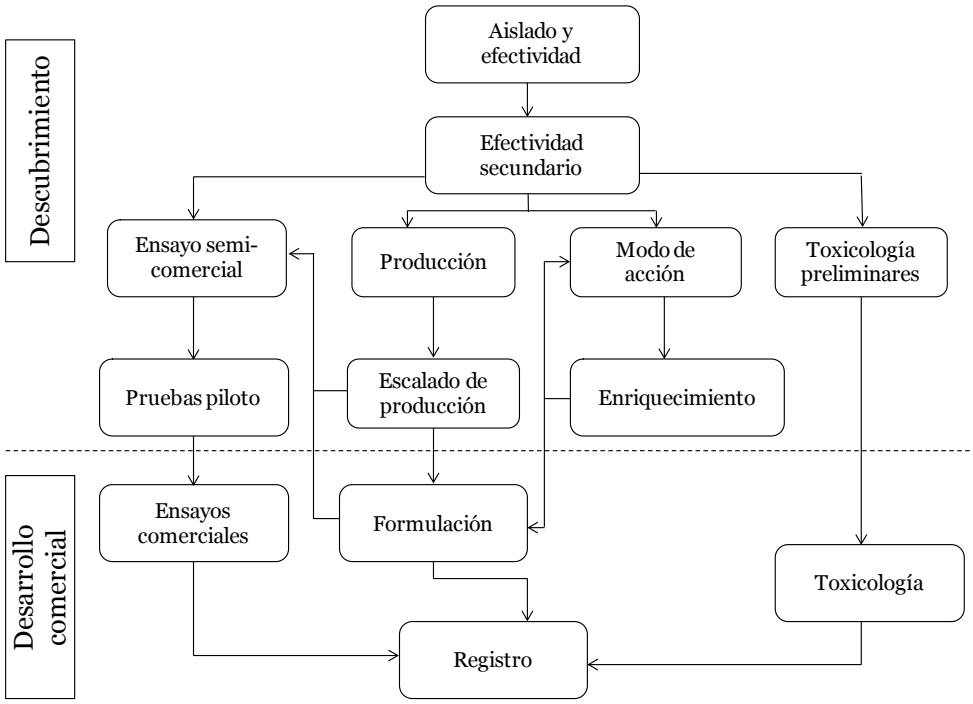


Figura 2. Diagrama de flujo del desarrollo de un agente de control biológico de postcosecha (Nunes, 2012).

Actualmente hay muy pocos productos que se estén comercializando a nivel mundial e incluso el mercado de algunos productos está limitado a unos cuantos países. Entre los productos de control biológico aplicados en postcosecha que en algún momento se han estado comercializando tenemos: BioSave™ es un producto a base del ACB *Pseudomonas syringae*, producido por Jet Harvest Solutions y comercializado en EE. UU.; Shemer™ a base de *Metschnikowia fructicola*, primero comercializado por Agrogreen más tarde por Bayer Crop Science y actualmente sublicenciado a Koppert y en fase final de registro en Europa; Serenade (*B. subtilis*, AgraQuest, EE. UU.); Boniprotect (*Aureobasidium pullulans*, Bio-ferm, Alemania) que ha sido registrado como un fortificante en Alemania y España; Yieldplus (*C. albidus*, Sudáfrica) comercializado primero por Anchor Biotechnologies y actualmente por LALLEMAND Plant Care Unit, NEXY (*C. oleophila*, BioNext, Bélgica) y Candifruit (*C. sake*, España) comercializada por SIPCAM INAGRA a demanda durante dos campañas y actualmente fuera del mercado y con los derechos de explotación en manos del IRTA.

2.2. Formulación de un agente de control biológico

Como se ha mencionado anteriormente, la formulación del ACB es uno de los pasos esenciales para su desarrollo comercial. El objetivo de la formulación de un ACB es obtener un producto que mantenga la viabilidad del antagonista durante largos periodos de almacenamiento (vida útil), que sea eficaz contra los patógenos de forma similar al microorganismo en fresco, y fácil de manipular y aplicar (Rhodes, 1993).

Las formulaciones de los ACBs las podemos clasificar en formulaciones líquidas y sólidas. Las formulaciones líquidas consisten en separar la biomasa del medio de producción y re-suspenderla en un medio líquido que puede contener estabilizantes, adherentes, surfactantes, colorantes u otros nutrientes adicionales (Abadias *et al.* 2003). Este tipo de formulaciones necesitan normalmente ser distribuidas y almacenadas bajo temperaturas de refrigeración, lo cual representa un inconveniente para su uso práctico, además que tienen una vida comercial bastante corta (Melin *et al.* 2007). En cambio, las formulaciones sólidas pueden ser manipuladas a temperatura ambiente, y su vida útil en general puede ser más larga.

La deshidratación del producto y el mantenimiento en un entorno seco es una de las mejores maneras para formular agentes microbianos para que puedan ser manejados usando los canales de distribución y almacenamiento normales (Rhodes, 1993). Desafortunadamente, algunos microorganismos son susceptibles a la desecación y muchos tienden a perder viabilidad durante el proceso de secado y en el almacenamiento. Los procesos de deshidratación clásicos son: liofilización, secado por pulverización o atomización, y secado por lecho fluido. Estos procesos cambian el estado físico del agua mediante la variación de la temperatura o la presión, o bien, por la combinación de ambos parámetros. De esta manera, la eliminación del agua ralentiza el metabolismo de las células previniendo la acumulación de productos tóxicos y disminuyendo la desnaturalización de las proteínas (Santivarangkna *et al.* 2007b).

2.2.1. Técnicas de deshidratación

a) Liofilización

Es la técnica más usada para la conservación de microorganismos, incluyendo colecciones tipo. Permite mantener la viabilidad de los microorganismos durante más de veinte años y no requiere condiciones especiales de baja temperatura para la conservación posterior (Prakash *et al.* 2013). El principio de este proceso consiste en la extracción del agua de células congeladas utilizando presión reducida (sublimación) dentro de una cámara de vacío. En este método el agua de las células congeladas pasa directamente del estado sólido a vapor mediante vacío. Generalmente, se usan sustancias

crioprotectoras como la leche, suero o glutamato de sodio. El nivel de la viabilidad después de la liofilización varía en función de numerosos factores, incluyendo el tipo de microorganismo, así como la eficacia del protector utilizado durante el proceso. Una ventaja adicional que poseen los cultivos liofilizados es que pueden ser distribuidos bajo condiciones de temperatura ambiente sin perder actividad (Navarta *et al.* 2011).

b) Atomización

Actualmente, la atomización es un método de secado muy estudiado para la formulación de bacterias probióticas (Peighambardoust *et al.* 2011; Schuck *et al.* 2013) y es de los métodos más económicos usado para producción y formulación industrial de cultivos probióticos (Santivarangkna *et al.* 2007a). El principio de la atomización se basa en producir un polvo seco a partir de una suspensión o cultivo microbiano líquido. El producto líquido es bombeado a una velocidad continua a través de una aguja inyectora o de un sistema centrífugo (atomizado) en forma de millones de microgotas (10–200 μm) dentro de una cámara de secado, donde es deshidratado usando aire caliente a elevadas temperaturas (entre 150–170 °C). Las microgotas atomizadas se secan instantáneamente en gránulos de polvo por acción del aire caliente que circula a alta velocidad dentro de la cámara y antes de que lleguen a la base son arrastradas al separador de polvo (ciclón) para ser recogidas en un depósito colector (Bhandari *et al.* 2008). Existen pocos estudios sobre el uso de la atomización para la formulación de ACBs (Abadias *et al.* 2005; Guijarro *et al.* 2006), y la mayoría con niveles de supervivencia bajos, debido a la utilización de altas temperaturas. Sin embargo, este método ha sido más efectivo para formulación de *B. subtilis* CPA-8 que es un microorganismo termo-resistente (Yánez-Mendizábal *et al.* 2012).

c) Lecho fluido

En este método de secado, el microorganismo mezclado con coadyuvantes en forma de pasta se extrusiona y deposita sobre bandejas perforadas a través de las cuales pasa una corriente de aire (35–45 °C) que fluidifica el producto produciéndose el secado. Las principales ventajas de este método son que se pueden obtener productos de baja humedad en corto tiempo y a bajos costes de mantenimiento con temperaturas de secado moderadas (Stummer *et al.* 2012). El uso del lecho fluido junto con la liofilización ha sido descrito con éxito en la conservación de ACBs como *Epicoccum nigrum* (Larena *et al.* 2003a), *Penicillium oxalicum* (Larena *et al.* 2003b) y *Penicillium frequentans* (Guijarro *et al.* 2006).

Estos procesos de deshidratación se caracterizan por una reducción de la actividad de agua, que se puede dar por la pérdida de agua libre o agua unida. La pérdida de agua libre provoca la disminución del volumen celular, precipitación de sales, oxidación de

compuestos y la detención del crecimiento celular. La pérdida de agua unida provoca daños irreversibles en la estructura celular y la pérdida de la viabilidad (Prakash *et al.* 2013).

2.3. Daños celulares durante un proceso de deshidratación

Entre los cambios que sufren las células durante un proceso de deshidratación destacan (Prakash *et al.* 2013):

1) *Cambios morfológicos*. Se produce la deformación de la célula debido a la pérdida del agua interna.

2) *Cambios en la presión osmótica*. Se produce un aumento de la presión osmótica de la célula como consecuencia de la pérdida de agua.

3) *Lesiones en la membrana*. La deshidratación puede provocar cambios en la membrana y pérdida total o parcial de sus funciones. Esto podría provocar pardeamientos enzimáticos o proteólisis por el simple hecho de que substratos y enzimas se ponen en contacto. Del mismo modo se pueden liberar sustancias perjudiciales a través de las membranas dañadas. En el probiótico *Bifidobacterium bifidum* BB-12 se describieron daños severos en la membrana, despolarización e incluso pérdida de la viabilidad durante el proceso de secado (Salar-Behzadi *et al.* 2013). Sin embargo, en otros casos las lesiones en la membrana no han sido críticas para el mantenimiento de la viabilidad del microorganismo, ya que una vez rehidratado utilizando medios de rehidratación adecuados, éste es capaz de reparar el daño y volver a crecer (Teixeira *et al.* 1997).

4) *Cambios bioquímicos* como serían: *(i)* disminución en la cantidad de fosfolípidos, *(ii)* alteraciones en el grado de saturación de los ácidos grasos, *(iii)* reacciones de peroxidación, que pueden llevar a la destrucción de los lípidos de la membrana y *(iv)* pérdida de la estructura terciaria de las proteínas (Santivarangkna *et al.* 2008).

5) *Daño en el material genético*. El daño en el genoma se debe principalmente a la acumulación de mutaciones durante el tiempo en el cual la bacteria no crece debido al estado de deshidratación. El daño puede producirse a través de modificaciones químicas (alquilación u oxidación), entrecruzamiento de bases (depurinización) y ruptura del ADN (Biegerdose *et al.* 1992); aunque es posible que sea reparado después de la rehidratación (Potts, 1994).

Durante la rehidratación, el agua entra en las células bruscamente hasta que se equilibra la presión osmótica. Las bacterias gram-negativas pueden sufrir lisis osmótica, debido a que tienen pared celular débil o bien, sufrir pérdida de electrolitos, carbohidratos, aminoácidos y nucleótidos a través de la membrana (Disalvo *et al.* 2008).

3. REGISTRO Y NORMATIVA

Como ya hemos mencionado, el desarrollo comercial es una de las fases más complejas incluida en el programa de desarrollo de un ACB, e implica varios pasos entre los que se incluye estudios de riesgos ambientales, exigidos para el proceso de registro y otros aspectos regulatorios (Nunes, 2012; Teixidó *et al.* 2011b).

En cuestión de registro y aspectos regulatorios son pocos los países que, como EE. UU., disponen de normativas específicas y simplificadas para el registro de este tipo de productos biológicos. En otros países, como los de la Unión Europea, el registro se ve limitado por la falta de una reglamentación específica que reconozca plenamente sus diferencias fundamentales con respecto a los productos fitosanitarios de síntesis. A nivel europeo, los productos empleados para la protección vegetal se regulan de acuerdo con el reglamento nº 1107/2009, relativo a la comercialización de los productos de protección vegetal, que actualiza la normativa existente y sustituye a las Directivas 91/414/CEE y 2005/25/CE.

Los principios para la evaluación y autorización de los productos fitosanitarios (químicos y biológicos) se incluyen en el Reglamento (CE) Nº 546/2011 y marcan las pautas a seguir para el desarrollo de un producto biológico. Los requisitos de evaluación están basados en unos principios generales y específicos. Los principios generales determinarán o evaluarán el rendimiento (eficacia y fitotoxicidad para cada uso), en los que se tendrán en cuenta las condiciones agrícolas, fitosanitarias, climáticas y medioambientales de las áreas de uso y la peligrosidad (riesgos probables para personas, animales y medio ambiente). En este último punto es donde surgen las mayores diferencias (y ventajas) para un ACB frente a una sustancia sintética ya que se trata de microorganismos que normalmente se encuentran en el medio ambiente (Droby *et al.* 2009; Sundh y Goettel, 2013).

Entre los principios específicos se incluye que se deberá: *(i)* evaluar el destino y comportamiento en el medio ambiente teniendo en cuenta la biocomplejidad de los ecosistemas, *(ii)* las interacciones en las comunidades microbianas y *(iii)* el potencial de persistencia y multiplicación de los microorganismos en todos los compartimentos ambientales.

Además, los estados miembros evaluarán los métodos analíticos propuestos para el control y seguimiento de los componentes viables e inviables, tanto en la formulación como en los residuos presentes en el interior y en la superficie de cultivos tratados. Para lo cual, es necesario determinar y validar adecuadamente los métodos que nos permitan identificar y/o cuantificar al ACB del resto de la microbiota.

4. MÉTODOS DE DETECCIÓN Y CUANTIFICACIÓN DE MICROORGANISMOS

Un gran número de métodos de detección y cuantificación han sido desarrollados para el seguimiento y monitorización de microorganismos. La elección del método está determinada por el nivel de especificidad que exige el estudio (familia, subfamilia, género, especie o cepa). En el caso de los ACBs, la detección específica a nivel de cepa es muy importante, debido a que cuando son aplicados en fruta, ya sea en pre- o postcosecha, éstos son liberados al medio ambiente donde la comunidad microbiana es sumamente compleja y además normalmente hay presentes otras cepas pertenecientes a la misma especie del ACB. Y es importante en muchos casos poder distinguir el ACB aplicado del resto de microorganismos presentes.

A pesar de la gran variedad de métodos de detección descritos en la literatura para el estudio de microorganismos en la naturaleza (Girones *et al.* 2010; Li y Wu, 2008; van Elsas *et al.* 1998), nos centraremos en aquellos que han sido utilizados para monitorizar ACBs, entre los que encontramos los métodos directos, microbiológicos y moleculares.

4.1. Métodos directos

Los métodos directos de cuantificación de microorganismos permiten establecer la población total de microorganismos existentes en la muestra, sin necesidad de cultivar al microorganismo. Tienen la ventaja de ser rápidos aunque no es posible diferenciar las células vivas de las muertas. Entre estos métodos tenemos: *(i)* La turbidimetría que relaciona la turbidez o densidad óptica de una suspensión microbiana directamente con la cantidad de microorganismos y *(ii)* el recuento de células en el microscopio. Este método consiste en depositar un volumen conocido de muestra en un portaobjetos especial que en la mayoría de los casos están excavados y cuadriculados (hemocitómetro,

cámaras de Petroff-Hauser, Neubauer o de Helber) para facilitar el recuento por unidad de superficie y de volumen (Forbes *et al.* 2009).

4.2. Métodos microbiológicos

Los métodos microbiológicos, también conocidos como métodos basados en cultivos o clásicos, han sido utilizados habitualmente por la mayoría de investigadores para el seguimiento de ACBs (Cañamás *et al.* 2008a; Cañamás *et al.* 2008c; De Cal *et al.* 2009; Teixidó *et al.* 1998), siendo el recuento en placa el más utilizado por ser una técnica fácil y económica de realizar.

La técnica de recuento en placa se basa en el supuesto de que cada bacteria crece y se divide para producir una sola colonia. Aunque este criterio no siempre se cumple porque con frecuencia las bacterias crecen unidas ya sea formando cadenas o aglomerados. Por consiguiente, a menudo una colonia no se produce como resultado de una única bacteria sino de segmentos cortos de una cadena o de un agregado bacteriano. Para reflejar esta realidad, los recuentos en placa suelen expresarse como unidades formadoras de colonias (UFC). Cuando se realiza el recuento en placa es importante que crezca un número limitado de colonias, para evitar inexactitudes en el recuento. Para esto es necesario diluir varias veces el inóculo inicial, y a este proceso se le denomina dilución seriada (Tortora *et al.* 2007). El máximo requerimiento de estos métodos es la disponibilidad de un medio selectivo o semi-selectivo en el cual sólo la cepa de interés pueda crecer y ser fácilmente diferenciable de otras. Generalmente, cuando no se cuenta con un medio selectivo, se suele recurrir a la diferenciación por características fenotípicas de la cepa para monitorear al ACB.

Una de las ventajas de esta técnica es que cuantifica las células viables. En cambio, tiene como desventajas: *(i)* que requiere de bastante tiempo, por lo general 24 h o más, para que se formen colonias visibles, y *(ii)* que no detecta aquellas células dañadas que son metabólicamente activas, pero no cultivables (en estado viable pero no cultivable (VBNC del inglés: *viable but nonculturable*)). Otra desventaja de esta técnica es que puede dar lugar a una identificación equívoca, debido a que no permite discriminar al ACB de otras cepas con el mismo fenotipo y que pertenecen a la microbiota de las plantas y/o fruto. En consecuencia, esta técnica no es del todo adecuada para la realización de estudios de seguimiento, persistencia e impacto ambiental después de la aplicación del antagonista (Schena *et al.* 2004). Por ello, se está recurriendo cada vez más al uso de métodos altamente específicos y fiables, como son los moleculares.

4.3. Métodos moleculares

En la actualidad, las técnicas moleculares para la identificación de ACBs están teniendo un auge muy importante debido a: *(i)* la alta especificidad que presentan (pueden detectar sólo la molécula o microorganismo de interés); *(ii)* su sensibilidad (son capaces de detectar la presencia de un solo microorganismo); y *(iii)* su rapidez (se puede identificar un microorganismo en menos de 24 horas). Además, el uso de técnicas moleculares permite estudiar las poblaciones microbianas sin hacer aislamientos, por tanto, se evitan los sesgos que pueden surgir con el método basado en el cultivo de microorganismos (Babalola, 2003).

Existe una amplia variedad de técnicas moleculares que se han propuesto para la detección de microorganismos de interés en diferentes áreas, que van desde la detección de la microbiota en suelo (van Elsas y Boersma, 2011), hasta la caracterización e identificación molecular de levaduras en bebidas fermentadas (Bokulich *et al.* 2012). Sin embargo, nos vamos a centrar únicamente en los métodos moleculares basados en la detección de los ácidos nucleicos mediante la reacción en cadena de la polimerasa (PCR del inglés: *polymerase chain reaction*), que es uno de los temas centrales de esta tesis.

5. REACCIÓN EN CADENA DE LA POLIMERASA (PCR)

La PCR es una técnica que permite amplificar secuencias de ADN *in vitro* por repetición de la reacción de elongación a partir de cebadores específicos, con una ADN polimerasa, comúnmente conocida como *Taq* Polimerasa. La *Taq* polimerasa es una enzima que, en unas condiciones determinadas y en presencia de una pequeña cadena de ADN es capaz de producir millones de copias de determinados fragmentos del ADN (Figura 3).

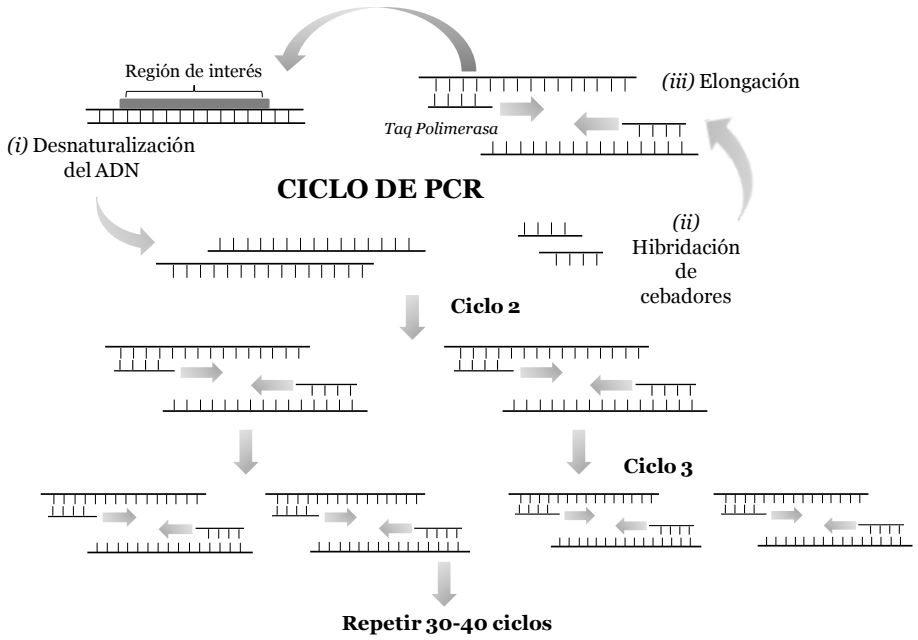


Figura 3. Ciclo de la reacción en cadena de la polimerasa (PCR)

La PCR se fundamenta en la repetición de tres pasos: *(i)* en el primer paso, lleva a cabo la desnaturalización de la cadena bicatenaria de ADN para producir dos moléculas monocatenarias; *(ii)* en un segundo paso, se realiza la hibridación de los cebadores complementarios a los extremos de las secuencias de ADN a amplificar, y *(iii)* en el tercer paso, se inicia la elongación o síntesis de nuevas cadenas mediante la acción de la *Taq* polimerasa. Posteriormente, estos productos se desnaturalizan, como se indica en el primer paso, y se repite el proceso para obtener múltiples copias de un segmento de ADN (Tagu y Moussard, 2006). Estos fragmentos se separan posteriormente por peso molecular y conformación mediante técnicas electroforéticas, obteniéndose un patrón de bandas específico que nos permite diferenciar individuos de la misma especie y entre mutantes derivados de la misma cepa parental (Carvalho, 1998; Schena *et al.* 2000).

La técnica PCR ha suministrado un conjunto de marcadores, tales como: los RAPDs, REP, ERIC, AFLPs, SAMPLs, amplificación directa con ADN microsatélites (DAMD), entre otros (Montesinos *et al.* 2008). Los polimorfismos en la longitud de fragmentos amplificados, más conocidos por su acrónimo inglés AFLP (*Amplified fragment length polymorphism*), son un tipo de marcador molecular que ha sido aplicado para el seguimiento de microorganismos antagonistas, tales como *Rhodotorula glutinis*, *C. laurentii, A. pullulans* tanto en campo como en condiciones de almacenamiento en frío (Lima *et al.* 2003). También se desarrollaron para la diferenciación del antagonista *Metschnikowia pulcherrima* (Spadaro *et al.* 2008). En todos los casos, los autores coinciden que este tipo de marcadores son altamente reproducibles; pero que su análisis es muy laborioso y costoso. Por lo que otros investigadores se han declinado por el diseño de un marcador basado en regiones amplificadas caracterizadas por secuencia (SCAR del inglés: *specific-characterized-amplified-region*), ya que dan lugar a una amplificación reproducible de un fragmento único y específico de ADN, el cuál diferencia a la cepa diana de otras cepas (Schena *et al.* 2002).

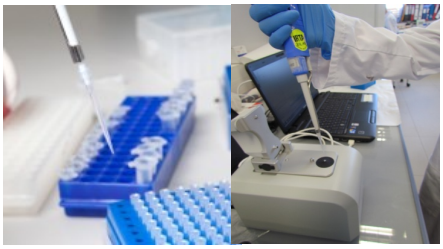
El uso de los marcadores SCARs se ha convertido en el enfoque más novedoso para monitorizar y seguir a un microorganismo en el medio ambiente, ya que permite distinguir al ACB de otras cepas de la misma especie presentes en el medio ambiente con una alta sensibilidad y reproducibilidad (Alabouvette y Cordier, 2011). Por lo que han sido utilizados para el marcaje de algunos ACBs, tales como: *P. anomala* cepa K (De Clercq *et al.* 2003), *B. subtilis* BD170 (Broggini *et al.* 2005), *Pseudomonas fluorescens* EP62e (Pujol *et al.* 2005), *P. oxalicum* 212 (Larena y Melgarejo, 2009a) y *Bacillus cereus* cepa TS02 (Chen *et al.* 2010). Una vez secuenciados y caracterizados los fragmentos SCARs se pueden diseñar cebadores específicos para ser utilizados en una amplificación simple definida mediante PCR.

5.1. PCR convencional

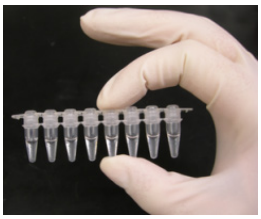
El proceso de detección de un ACB por amplificación genética se desarrolla de manera habitual en tres etapas (Figura 4). La primera etapa consiste en la extracción y purificación del ADN del microorganismo de la muestra, seguido de la amplificación de un segmento seleccionado del genoma del microorganismo mediante PCR. Finalmente, en la tercera etapa se lleva a cabo la detección de los fragmentos amplificados en la PCR (amplicones) por electroforesis en gel de agarosa y tinción con bromuro de etidio. Una vez teñido, al exponer el gel a la luz ultravioleta, los electrones del bromuro de etidio se estimulan produciendo fluorescencia, pudiéndose así visualizar los fragmentos de la secuencia de ADN que hemos amplificado. En general todo este proceso suele durar aproximadamente 24 h (Tagu y Moussard, 2006).

Una de las ventajas de la PCR convencional es su alta especificidad y sensibilidad para detectar microorganismos, siendo una herramienta útil para la detección de varios ACBs tales como: bacterias (Chen *et al.* 2010; Felici *et al.* 2008; Holmberg *et al.* 2009; Pujol *et al.* 2005), levaduras (De Clercq *et al.* 2003; El Hamouchi *et al.* 2008; Schena *et al.* 2002) y hongos (Feng *et al.* 2011; Hermosa *et al.* 2001; Naeimi *et al.* 2011). Sin embargo, no permite llevar a cabo un análisis cuantitativo. Por ello, se han diseñado técnicas basadas en la PCR complementados con métodos microbiológicos como: recuento en placa en medios selectivos, para la detección y cuantificación de dos cepas de *A. pullulans* 1-1 y 1113-5 (El Hamouchi *et al.* 2008), *P. anomala* K en la superficie de manzana (De Clercq *et al.* 2003), o bien, para la monitorización de *P. fluorescens* EPS62e en campo (Pujol *et al.* 2005).

Etapa 1. Extracción y purificación del ADN



Etapa 2. Amplificación mediante PCR



Etapa 3. Electroforesis en gel de agarosa y visualización de los productos de amplificación

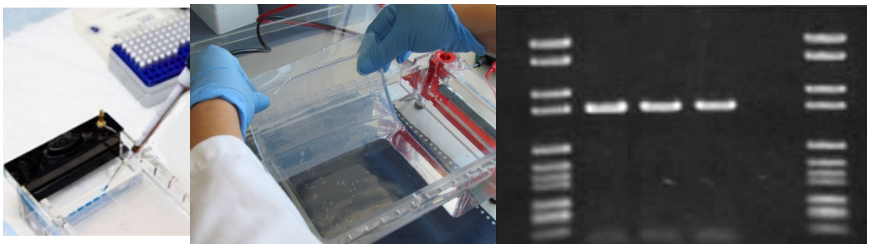


Figura 4. Pasos para el desarrollo de la PCR convencional

No obstante, esta técnica aún presenta una de las desventajas de los métodos microbiológicos que es subestimar el tamaño de la población debido a que el microorganismo puede entrar en un estado VBCN. Esta limitación puede resolverse determinando los niveles poblacionales totales (cultivables y no cultivables) mediante la PCR cuantitativa o a tiempo real (qPCR).

5.2. PCR a tiempo real (qPCR)

En la qPCR, los procesos de amplificación y detección se producen de manera simultánea en el mismo vial cerrado, sin necesidad de ninguna acción post-PCR (electroforesis). Los termocicladores que se utilizan para realizar la qPCR incorporan un lector de fluorescencia y están diseñados para poder medir, a tiempo real, la fluorescencia emitida en cada uno de los viales donde se realice la amplificación.

La qPCR se basa en el seguimiento ciclo por ciclo de la reacción de amplificación del ADN blanco, mediante el uso de detectores, los cuales serán descritos más adelante, que permiten detectar a tiempo real la cantidad de producto amplificado. De modo que a medida que aumentan los ciclos de PCR, aumenta también la intensidad de fluorescencia emitida. Lo que hace posible trazar una curva de amplificación a lo largo del progreso de la reacción de PCR (Higuchi *et al.* 1992).

La curva de amplificación consta de tres partes, tal y como se muestra en la Figura 5a. La primera de ellas es una fase exponencial en la que, si la eficiencia de la reacción es del 100 %, se produce en cada ciclo exactamente el doble de producto que en el ciclo anterior. En esta fase la reacción es muy específica y precisa. A continuación tiene lugar la fase lineal, en la que alguno de los reactivos puede ser limitante, la *Taq* polimerasa pierde actividad o bien alguno de los productos comienza a degradarse. La reacción es más lenta y por lo tanto aumenta la variabilidad en la cantidad del producto final. Finalmente se produce una fase de meseta ("*plateau*"), en la que existe inhibición por producto y la reacción de síntesis se detiene (Kainz, 2000).

El parámetro fundamental en una qPCR y en función del cual se van a realizar todos los cálculos analíticos y la obtención de resultados, es el denominado Ciclo Umbral (del término en inglés: *Threshold Cycle* o *Cq*), que se define como el ciclo a partir del cual la fluorescencia es significativa por encima del ruido de fondo (*background*). El valor *Cq* puede considerarse como la unidad de medida básica de la qPCR (Higuchi *et al.* 1992). El *Cq* se determina en la fase exponencial de la reacción de PCR, (Figura 5b), y es inversamente proporcional al número de copias del ADN molde. Por lo tanto, cuanto más alto es el número de copias iniciales de los ácidos nucleicos a amplificar, antes se observa un aumento significativo en la fluorescencia, y son más bajos los valores de *Cq*.

La principal característica que define la qPCR a tiempo real es la posibilidad de obtener resultados cuantitativos. El análisis cuantitativo de ácidos nucleicos puede realizarse de dos formas, mediante cuantificación relativa o absoluta. La cuantificación relativa permite determinar los cambios en la expresión de un gen de una muestra con respecto a un estándar externo o la expresión de un gen de referencia (Livak y Schmittgen, 2001). La aplicación más utilizada, de la cuantificación relativa, es la comparación de los niveles de expresión génica (ARNm) entre diferentes tejidos, en un mismo tejido a lo largo del tiempo, o la respuesta de un tejido a diferentes tratamientos. En cambio, el objetivo de la cuantificación absoluta es determinar el número exacto de moléculas de ADN presentes en una muestra y para ello se emplea una curva de calibración. El resultado estará expresado en las mismas unidades que los estándares de la curva de calibración (número de copias/mL, ng/mL entre otros). La cuantificación

absoluta se emplea para la detección y cuantificación de microorganismos, tales como los ACBs.

En la cuantificación absoluta, la concentración inicial de ADN diana en la muestra se puede cuantificar de manera muy sencilla, creando una curva estándar o patrón (Figura 5c). La curva estándar o patrón se realiza mediante la amplificación de diluciones seriadas de un estándar de concentración conocida. En cada ciclo de PCR se registra el incremento de fluorescencia que es proporcional a la cantidad de ADN del estándar y se representa el logaritmo decimal de las concentraciones de los estándares versus el *Cq* (Livak y Schmittgen, 2001). El resultado es una recta $y = -m$ Log $(x)+b$, cuya pendiente (m) deberá ser teóricamente igual a −3.32 si tenemos una eficiencia de amplificación del 100 % ($E= 10^{-1/m}$). En la práctica, un ensayo es aceptable hasta con un 92 % de eficiencia. Una vez construida la curva estándar la concentración de ADN de la muestra se calcula interpolando en ella los valores de los *Cq* de cada muestra problema.

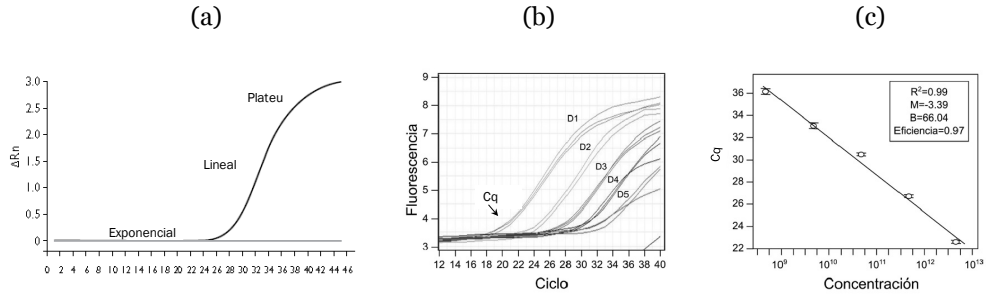


Figura 5. Parámetros de la PCR cuantitativa (qPCR): a) curva de amplificación, b) Ciclo de umbral (*Cq*) y c) curva estándar. Se presentan los valores del coeficiente de correlación (R^2), la pendiente (M), el intercepto (B) y la eficiencia de la reacción (E).

En el momento de diseñar un ensayo de qPCR han de tenerse en cuenta diversas variables, por ejemplo, el grado de especificidad y sensibilidad que el estudio requiera, para lo que es importante elegir algún sistema que permita detectar la amplificación del producto de PCR.

5.2.1. Sistemas de detección

Los sistemas de detección por fluorescencia empleados pueden ser de dos tipos: inespecíficos y específicos (Wong y Medrano, 2005).

a) Sistemas de detección inespecíficos

Los sistemas de detección inespecíficos se basan en el uso de agentes intercalantes de ADN que se unen al surco menor del ADN bicentenario. Tras esta unión se produce un cambio de conformación que da lugar a un incremento de la fluorescencia. Entre ellos se encuentran los fluorocromos como el bromuro de etidio (Higuchi *et al.* 1992), el YOPRO® y YoYo® de Molecular Probes (Ishiguro *et al.* 1995; Ogura y Mitsuhahi, 1994) y el SYBR® Green I (Becker *et al.* 1996); el cual es uno de lo más demandados por su facilidad de utilización y disponibilidad comercial; y BEBO es de las últimas moléculas intercalantes descritas, y comparte características en común al SYBR® Green I (Figura 6) (Bengtsson *et al.* 2003).

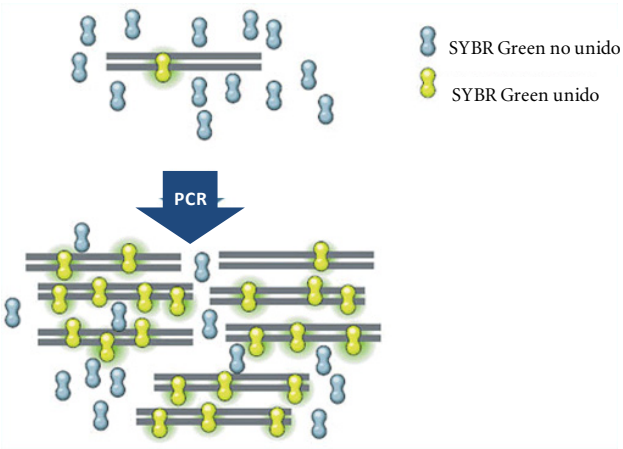


Figura 6. Detección de la amplificación mediante agentes intercalantes fluorescentes (SYBR® Green). El colorante se une al surco menor del ADN bicatenario, pero no al ADN monocatenario. Como consecuencia de esta unión, se produce un incremento de la señal fluorescente (representado en color verde). Debido al aumento de la cantidad de ADN recién sintetizado tras cada ciclo del PCR, la emisión de fluorescencia aumenta en proporción al número de moléculas del DNA bicatenario presentes en la mezcla.

El principal inconveniente de los agentes intercalantes es que no dan información sobre la especificidad de la reacción; es decir, no distinguen entre los productos específicos de la reacción de los inespecíficos (como los dímeros de oligonucleótidos), con lo que existe la probabilidad de detección de falsos positivos (Wittwer *et al.* 1997). Por ello, el uso de las curvas de fusión de los productos de PCR se realiza para diferenciar productos específicos de los inespecíficos. Para ello se estudia la temperatura de fusión del producto específico (conocida como *Tm* que es la temperatura a la que el 50 % de la molécula de ADN está desnaturalizada) y de esta forma asegurar la amplificación y cuantificación únicamente del producto de interés (Costa, 2004).

b) Sistemas de detección específicos

Por otro lado, los sistemas de detección específica utilizan sondas que son una secuencia de oligonucleótidos específicos homóloga al producto diana, marcada con un fluoróforo en cada extremo del oligonucleótido, para cuantificar únicamente el producto de interés (Montesinos *et al.* 2008).

De manera general las sondas específicas se pueden clasificar en dos grandes grupos: *(i)* sondas de hidrólisis (*Taqman*® y MGB), y *(ii)* sondas de hibridación (Molecular Beacons, Scorpion, FRET).

(i) Sondas de hidrólisis

La sonda de hidrólisis más utilizada es la llamada *Taqman* (Heid *et al.* 1996; Livak *et al.* 1995) consiste en un oligonucleótido marcado en su extremo 5' con un fluorocromo de alta energía, llamado "*reporter*" (FAM, TET, TAMRA, HEX, JOE, ROX, CY5 y Texas Red) y con otro fluorocromo de baja energía en el extremo 3´ llamado "*quencher*" (TAMRA, DABCYL y Methyl Red). El marcaje de la sonda con los dos fluorocromos aprovecha el fenómeno FRET (*fluorescence resonance energy transfer*) para conseguir una señal fluorescente específica cuando la sonda se une a la secuencia diana de ADN. Este mecanismo consiste en que cuando se produce la excitación de un fluorocromo de alta energía que está próximo a otro de baja energía se produce una transferencia de la misma de uno al otro. Por lo tanto, si una fuente de luz incide sobre la sonda, el "*quencher*" suprime la emisión de fluorescencia del "*reporter*" por su proximidad. Si se produjera una separación de ambos fluorocromos dejaría de producirse el fenómeno FRET y el "*reporter*" incrementaría la emisión de fluorescencia disminuyendo la del "*quencher*". Esto es lo que ocurre durante la fase de elongación, cuando la *Taq* polimerasa se encuentra con la sonda se produce la hidrólisis de ésta debido a la actividad 5' exonucleasa de la polimerasa (Figura 7) (Holland *et al.* 1991). Como la sonda sólo se hidroliza cuando se ha unido a la secuencia diana, la fluorescencia registrada procede únicamente de la amplificación específica del producto de PCR de interés (Forbes *et al.* 2009).

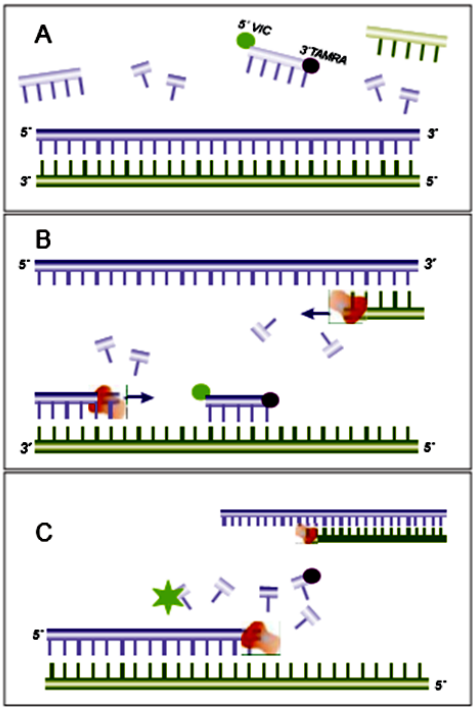


Figura 7. Mecanismo de acción de sondas de hidrólisis tipo *Taqman* en la qPCR. **A**. La reacción contiene inicialmente los cebadores, nucleótidos y la sonda marcada en 5' con un fluorocromo (VIC) y en 3' con un amortiguador de fluorescencia (TAMRA). **B**. La sonda y los cebadores se unen al ADN diana y se inicia al síntesis de la cadena de ADN. **C**. La actividad 5'- 3' exonucleasa de la *Taq polimerasa* hidroliza la sonda en su avance para generar el producto de amplificación. Al separarse los fluoróforos se produce la consiguiente emisión de fluorescencia. Como consecuencia, el número de moléculas que emiten fluorescencia aumenta en la misma medida que se produce la amplificación del DNA diana.

El uso de las sondas *Taqman* requiere que su *Tm* sea 10 °C por encima de la *Tm* de los cebadores y para cumplir este requisito la sonda debe de contener aproximadamente 27 nucleótidos. Esto puede suponer una dificultad cuando se desea diferenciar entre microorganismos de la misma especie, ya que puede resultar difícil encontrar una región conservada de ADN, en la que se den todos los requisitos para que los cebadores y la sonda se encuentren espaciados adecuadamente (Salmon *et al.* 2002). Para mejorar la especificidad de las sondas *Taqman* se desarrollaron las sondas *Taqman*-MGB, que mantienen la misma función y diseño que una sonda *Taqman* con alguna modificación. En la sondas *Taqman*-MGB se ha incorporado en su extremo 3' una molécula denominada MGB (*Minor Groove Binder*) que se une al surco menor de la doble hebra de ADN.

Esta molécula, tripéptido de dihidrociclopirroloindol (DPI3), se ensambla en la doble hebra formada por la sonda y la secuencia diana, dando lugar a una hibridación más estable (Figura 8). Además, provoca un aumento en la *Tm* de la sonda, dando lugar a la posibilidad de diseñar sondas de menor tamaño con una *Tm* más alta. Por ejemplo, una sonda MGB de 12 nucleótidos tendría una *Tm* de 65 °C, que es común para una sonda *Taqman* de 27 nucleótidos. La ventaja de las sondas *Taqman*-MGB reside en que se consigue mayor especificidad de secuencia al utilizar sondas más cortas. Su limitante, es la dificultad de diseño y la disminución de la eficiencia en amplicones mayores de 150 pb, además de implicar un coste elevado (Afonina *et al.* 1997; Kutyavin *et al.* 2000).

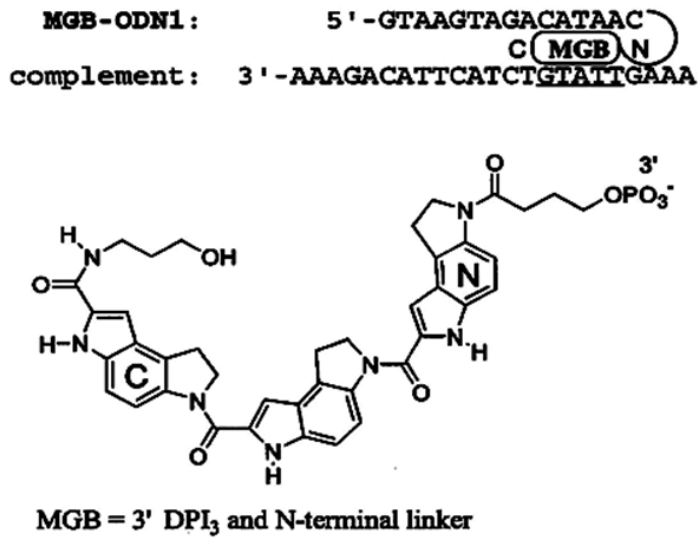


Figura 8. Esquema del dúplex de DNA formado con la sonda MGB y un oligonucleótido de 23 bases. La región que se encuentra subrayada corresponde a la zona en la que el tripéptido dihidrociclopirroloindol DPI3 encaja en el surco menor del DNA bicatenario. C y N indican la posición de los extremos carboxilo y amino del tripéptido. Debajo se muestra la estructura de la molécula DPI3 (Kutyavin *et al.* 2000).

(ii) Sondas de hibridación

Las sondas de hibridación emiten fluorescencia únicamente cuando hibridan con la molécula diana (Heid *et al.* 1996). Estas sondas están compuestas por oligonucleótidos de entre 15 y 30 pares de bases complementarios a una secuencia diana. En sus extremos presentan unas secuencias de entre 5 y 6 pares de bases, las cuales a su vez tienen unido covalentemente un "*reporter*" y un "*quencher*", dando lugar a una estructura secundaria

con forma de horquilla. Con esta estructura el "*reporter*" y el "*quencher*" se mantienen muy próximos por lo que se produce el fenómeno FRET y no se detecta fluorescencia hasta que se hibrida con su secuencia diana y adopta una conformación lineal. Al linearizarse, el "*reporter*" y el "*quencher*" se alejan y se produce la emisión de fluorescencia. El inconveniente de usar este tipo de sondas radica en la mayor dificultad en el diseño y la optimización, hay que tener en cuenta el tamaño y secuencia de la sonda para formar un híbrido estable, además de las secuencias de los extremos para que sean complementarias y estables cuando no esté la secuencia diana presente (Bernard y Wittwer, 2000).

Como hemos mencionado, la validación y el desarrollo de la qPCR es un proceso que implica varios pasos; tales como: la identificación de regiones diana, la elección de un enfoque de cuantificación absoluta y/o relativa, la elección de los detectores, validación de los cebadores y sondas específicas, el análisis de la sensibilidad, entre otros. Muchos de estos pasos son críticos para la validación de la técnica, todos ellos son analizados de manera exhaustiva en una revisión hecha por Schena *et al.* (2013), donde además exponen los errores más comunes que se pueden cometer durante el desarrollo de la qPCR.

5.2.2. Aplicación de la qPCR en estudios con ACBs

La qPCR ha demostrado su versatilidad y utilidad para una serie de aplicaciones en diferentes áreas de investigación, tales como: en microbiología de alimentos (Ceuppens *et al.* 2014; Postollec *et al.* 2011), en patologías de plantas (Sanzani *et al.* 2014) y en la detección y cuantificación de microorganismos antagonistas, aunque de una manera más limitada, como en: *A. pullulans* L47 y L30 (De Curtis *et al.* 2004; Schena *et al.* 2002), *C. oleophila* O (Massart *et al.* 2005), *C. oleophila* (Spotts *et al.* 2009), *C. infirmominiatum* (Spotts *et al.* 2009), *P. oxalicum* (Larena y Melgarejo, 2009b) y *Fusarium oxysporum* agente F047 (Edel-Hermann *et al.* 2011).

Algunos de estos estudios han mostrado una alta correlación de la qPCR con otros métodos de cuantificación (Massart *et al.* 2005; Spotts *et al.* 2009). Sin embargo, la qPCR sobreestimó la población viable del antagonista *P. fluorescens* EPS62e NaL en hojas de manzano (Pujol *et al.* 2006). Los autores lo atribuyen a la presencia de células en estado VBNC o bien, al ADN presente después de la muerte celular. Demostrando como principal inconveniente, la incapacidad de la qPCR para diferenciar entre células vivas y muertas.

6. DETERMINACIÓN DE LA VIABILIDAD CON MÉTODOS MOLECULARES

En el caso de ACBs no sólo es importante detectar la presencia del antagonista, sino que resulta crucial conocer si se trata de formas viables, ya que su eficacia para controlar la enfermedad está relacionada con la concentración de células viables en la suspensión del tratamiento, y su supervivencia en la superficie de la fruta (Chand-Goyal y Spotts, 1996).

Algunas investigaciones han hecho uso del ARNm para abordar la detección de células viables (Keer y Birch, 2003). Una estrategia se basó en una reacción de PCR con transcripción inversa, RT-PCR para detectar la viabilidad de *Listeria monocytogenes* (Klein y Juneja, 1997). Otra estrategia fue la técnica de amplificación isotérmica de ARN o técnica de NASBA (de sus siglas en inglés: *Nucleic Acid Sequence Based Amplification*). Ésta se ha utilizado con éxito para la detección viable de *Salmonella* spp. (D'Souza y Jaykus, 2003), *Mycobacterium avium* subsp. *paratuberculosis* (Rodríguez-Lázaro *et al.* 2004), *L. monocytogenes* (Nadal *et al.* 2007), y *Vibrio cholerae* (Fykse *et al.* 2007). No obstante, una de las desventajas de estas técnicas es que el ARN es más lábil que el ADN. Su degradación puede ocurrir durante la manipulación y el almacenamiento de muestras, originando falsos negativos. Además, la cuantificación está vinculada con el número de moléculas de ARNm y algunas no se transcriben en las células en estado VBNC (Yaron y Matthews, 2002).

En cambio el ADN puede persistir durante un largo tiempo después de la muerte celular, lo que conduce a una sobreestimación en la cuantificación. A pesar de esta alta estabilidad, el ADN se puede considerar como una buena molécula para la detección y cuantificación específica de los ACBs. Durante la última década, se ha introducido otro enfoque que ha permitido diferenciar cuantitativamente entre bacterias viables y no viables, mediante el empleo de agentes intercalantes de ADN asociado a la PCR cuantitativa. El principal criterio para distinguir entre células viables y células dañadas de manera irreversible es la integridad de la membrana. Las células viables con

membranas intactas son impermeables al paso de determinados agentes intercalantes de ADN; sin embargo, penetran fácilmente en células que presentan daños en las membranas (Fittipaldi *et al.* 2012; Nocker y Camper, 2009; Nogva *et al.* 2003).

6.1. Agentes intercalantes de ácidos nucleicos

Una de las primeras estrategias utilizadas para la detección de células viables fue desarrollada por Nogva *et al.* (2000). En este estudio, tras tratamientos térmicos de inactivación, se eliminaba el ADN de las células muertas de *Campylobacter jejuni* utilizando DNAsas y posterior lavado, quedando protegido el ADN de las células viables por sus cubiertas celulares. Posteriormente, este mismo grupo de investigación desarrollaría un nuevo método, denominado EMA-PCR, que mejoraba al anterior pero basándose en el mismo principio. La EMA-PCR, consiste en la capacidad de penetración de la molécula de monoazida de etidio (EMA) en las células a través de las membranas citoplasmáticas dañadas. Tras una fotoactivación, la molécula de EMA se une covalentemente al ADN quedando inaccesible durante la reacción de PCR (EMA-PCR). Este enfoque ha sido probado con diferentes patógenos de transmisión alimentaria; tales como: *Escherichia coli* O157:H7, *L. monocytogenes*, *Campylobacter* (Nogva *et al.* 2003; Rudi *et al.* 2005), *Salmonella* (Guy *et al.* 2006) y *Vibrio vulnificus* (Wang y Levin, 2006). Sin embargo, el papel del EMA como agente discriminante de bacterias dañadas ha sido cuestionado recientemente, al considerar que también puede penetrar en células intactas de algunas especies bacterianas (Kobayashi *et al.* 2009; Nocker *et al.* 2006). Por este motivo, se ha propuesto la utilización de otro agente intercalante de ADN, llamado propidio de monoazida (PMA).

6.1.1. Propidio de monoazida (PMA)

El PMA es el resultado de la adición de un grupo azida al ioduro de propidio (PI) que se utiliza satisfactoriamente en la discriminación mediante fluorescencia de bacterias viables en el test comercial LIVE/DEAD *Bac*Light. El grupo azida, tras un período de foto-exposición, genera un radical nitreno que es el que se une covalentemente al ADN e impide su amplificación por PCR. Estudios recientes han demostrado que esta unión covalente insolubiliza el ADN de células dañadas, lo que da lugar a su pérdida durante la extracción del ADN genómico (Figura 9).

El PMA no unido que permanece libre en solución, se inactiva por fotólisis y se convierte en hidroxilamina al reaccionar con moléculas de agua, de modo que no puede unirse al ADN de células viables en la etapa posterior de lisis celular (Nocker *et al.* 2006).

A pesar que el PMA y el EMA comparten el mismo concepto como intercalantes de tinción, difieren en cuanto su permeabilidad a través de las membranas celulares. El

EMA es ligeramente más eficaz en la supresión de la señal que el PMA debido a su composición química. Sin embargo, el PMA es más eficaz para discriminar entre células viables de no viables, debido a que posee una carga positiva superior al EMA, lo que aumenta su impermeabilidad a la penetración en células con membranas intactas (van Frankenhuyzen *et al.* 2011).

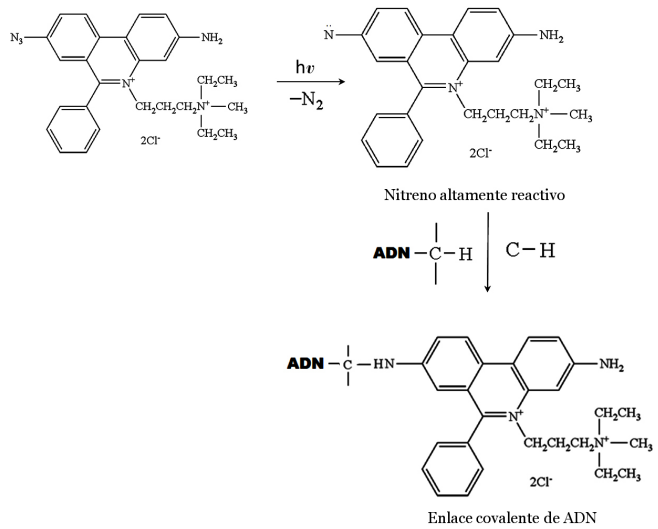


Figura 9. Principio del propidio de monazida (PMA) en la modificación del ADN

Antes de utilizar el PMA en combinación con la qPCR (PMA-qPCR) es necesario tener en cuenta varios aspectos técnicos, entre los que se destacan: *(i)* el tipo de microorganismo; en bacterias gram-negativas, la compleja estructura de la membrana representa una barrera mayor a la permeabilidad para el PMA que la barrera de peptidoglucano de gram-positivas (Nogva *et al.* 2003), *(ii)* la concentración del PMA y su efecto citotóxico, *(iii)* el tiempo de incubación y fotolisis, *(iv)* la matriz o bien, el proceso al que se halla sometido la muestra. Todas estas condiciones son discutidas exhaustivamente en la revisión realizada por Fittipaldi *et al.* (2012).

La técnica de PMA-qPCR ha sido probada con éxito para la detección selectiva y cuantificación de microorganismos en microbiología de alimentos, tal como lo muestra Elizaquível *et al.* (2014) en una revisión sobre el uso de esta técnica para la detección de patógenos de transmisión alimentaria en cultivo puro y en alimentos, tales como: *L. monocytogenes* (Pan y Breidt, 2007), *E. coli* O157:H7 (Elizaquível *et al.* 2012; Nocker y Camper, 2009), *C. jejuni* (Josefsen *et al.* 2010), *Salmonella* (Martin *et al.* 2013),

B. subtilis (Kim y Ko, 2012), así como en otros microorganismos relevantes en la industria alimentaria, como son la levaduras vinificantes (Andorra *et al.* 2010), y las bacterias ácido lácticas (Desfosses-Foucault *et al.* 2012) y para la identificación de microorganismos alterantes de alimentos (Crespo-Sempere *et al.* 2013). No obstante, se desconoce la aplicación de esta técnica para la cuantificación viable de ACBs.

7. ESTADO DEL ARTE DEL AGENTE DE BIOCONTROL *Pantoea agglomerans* CPA-2

El antagonista objeto de estudio de esta tesis es la bacteria *P. agglomerans* cepa CPA-2. ACB aislado de la superficie de manzana (Nunes *et al.* 2001a) y eficaz frente a las principales enfermedades de postcosecha en pera, manzana y cítricos (Nunes *et al.* 2001b; Teixidó *et al.* 2001). Desde su aislamiento hasta la fecha, este antagonista ha sido ampliamente estudiado por el grupo de Patología de la Postcosecha de centro IRTA-Lleida y de la Universidad de Lleida y prácticamente la totalidad de los pasos incluidos en el programa de desarrollo de un ACB se han llevado a cabo, tales como:

1) El aislamiento de la cepa en el laboratorio de Patología de la unidad de Postcosecha del IRTA-Lleida (Nunes *et al.* 2001c).
2) Estudios de efectividad tanto a nivel de laboratorio como en condiciones prácticas (Nunes *et al.* 2001a; Torres *et al.* 2007).
3) Se ha elucidado su modo de acción (Poppe *et al.* 2003; Torres *et al.* 2011).
4) Se ha mejorado su rango de actividad (Cañamás *et al.* 2011).
5) Se ha estudiado su compatibilidad con otros métodos alternativos (Plaza *et al.* 2004; Teixidó *et al.* 2001; Usall *et al.* 2008).
6) La optimización de la producción de la biomasa de forma económica (Costa *et al.* 2000a).
7) La formulación de este ACB con un razonable tiempo de conservación, manteniendo una vida útil eficaz (Costa *et al.* 2000b, 2001, 2002).
8) Se han estudiado varios enfoques para mejorar su comportamiento frente a condiciones de estrés y su viabilidad durante el proceso de formulación y mejorar su eficacia en aplicaciones en precosecha (Cañamás *et al.* 2008b, c; Teixidó *et al.* 2005, 2006).

9) El estudio de diferentes envases y condiciones de almacenamiento para mantener su estabilidad y prolongar la viabilidad de las células formuladas (Torres *et al.* 2014).
10) Además, se han desarrollado unos marcadores SCARs, PAGA1 y PAGB1, que permiten diferenciar molecularmente a CPA-2 de otras cepas con el mismo fenotipo, y pueden ser utilizados como herramienta para futuros estudios de seguimiento y persistencia en su entorno de aplicación (Nunes *et al.* 2008).

Para concluir, el programa de desarrollo de un ACB y generar la información que exige el proceso de registro para el futuro registro de Pantovital®, nombre comercial que se le ha dado a este agente de biocontrol, (BioDurcal S.L. Durcal, Spain) en Europa, es necesario realizar estudios que permitan demostrar el bajo riesgo medioambiental de *P. agglomerans* CPA-2; es decir, su poca persistencia en su entorno de aplicación, así como cuantificar el tamaño poblacional de este microorganismo sobre la fruta con metodologías adecuadas que nos permitan identificar y/o cuantificar a nuestra cepa de otras cepas con características fenotípicas similares pertenecientes a la microbiota del fruto. Y esto en definitiva es el principal objetivo de esta tesis.

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OBJETIVOS

OBJETIVOS

El principal objetivo de esta tesis es desarrollar técnicas moleculares para el seguimiento e identificación del agente de biocontrol *P. agglomerans* CPA-2, en su proceso de formulación y entorno de aplicación en pre- y postcosecha.

A continuación se detallan los objetivos específicos:

1) Desarrollar un sistema de qPCR usando sonda *Taqman*-MGB para la detección y cuantificación específica de *P. agglomerans* CPA-2.

a) Diseñar y optimizar los cebadores y la sonda *Taqman*-MGB para la cuantificación de CPA-2 mediante qPCR.
b) Evaluar la especificidad y eficiencia de los cebadores usando sonda *Taqman*-MGB.
c) Evaluar la fiabilidad de la técnica de la qPCR para el seguimiento poblacional de CPA-2 en la superficie de la fruta.

2) Desarrollar un método molecular para distinguir células viables y no viables de *P. agglomerans* CPA-2 combinando qPCR con PMA.

a) Optimizar la concentración del PMA, tiempo de incubación y tiempo de foto-exposición para CPA-2.
b) Evaluar la toxicidad del PMA en células de *P. agglomerans* CPA-2.
c) Optimizar la técnica en cultivo puro y en una matriz de naranja.

3) Aplicar la técnica PMA-qPCR para cuantificar células viables de *P. agglomerans* CPA-2 durante su formulación mediante liofilización, lecho fluido y atomización.

a) Determinar la supervivencia de distintos formulados de CPA-2 mediante las técnicas de recuento en placa, qPCR y PMA-qPCR.
b) Estudiar la efectividad de las células de CPA-2 cuantificadas mediante PMA-qPCR en estudios de efectividad en naranjas frente a infecciones de *P. digitatum*.

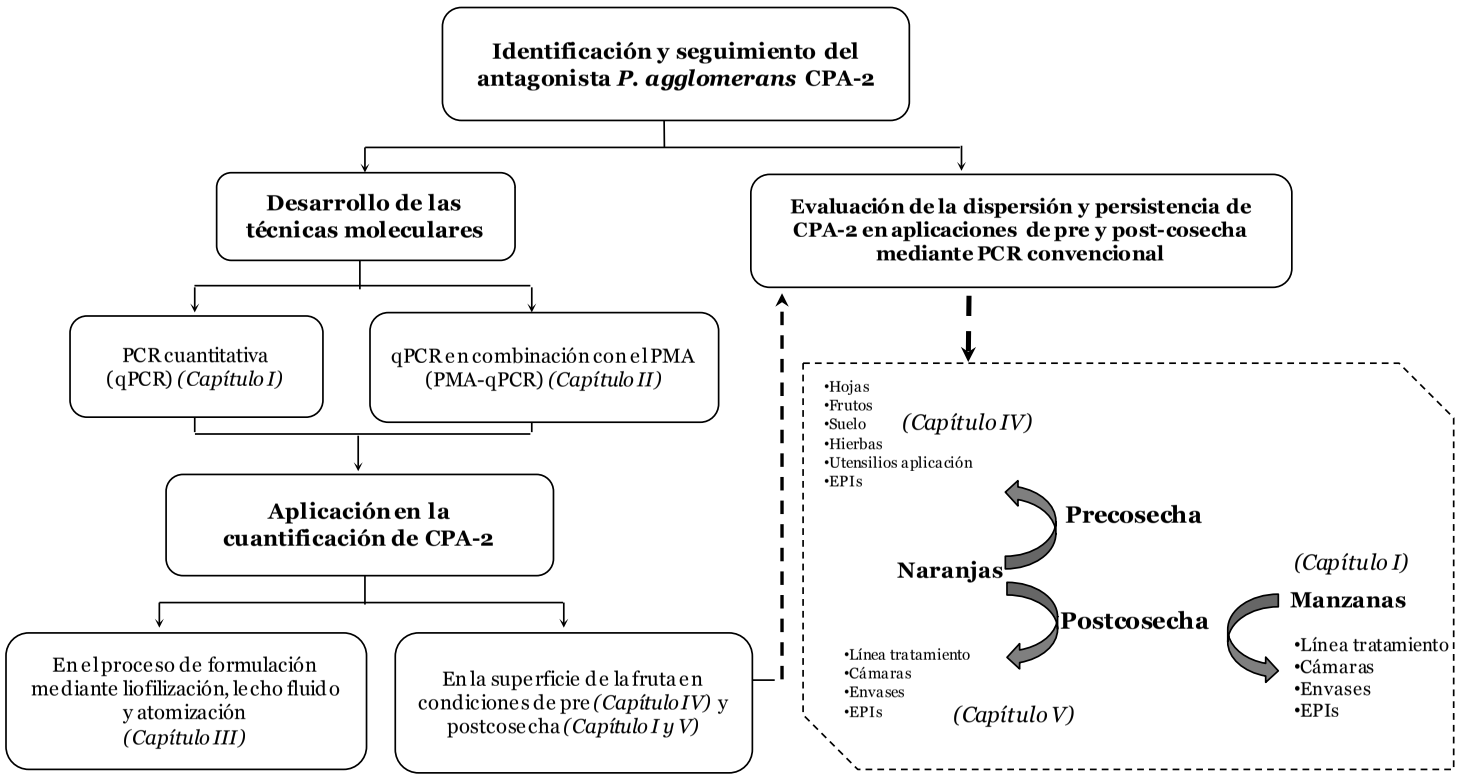
4) Estudiar la dispersión y persistencia de *P. agglomerans* en tratamiento de precosecha en naranja.

a) Estudiar la dispersión del tratamiento de CPA-2 aplicado en precosecha en campo en naranjas cv *Valencia Late* tratados mediante pulverización.
b) Estudiar la persistencia de CPA-2 en árboles tratados y no tratados, en la hierba, en el suelo; asimismo, sobre la ropa de los operarios y las herramientas de trabajo.

5) Evaluar la dispersión y persistencia de *P. agglomerans* CPA-2 en tratamiento de postcosecha en manzanas y naranjas

a) Estudiar la dispersión y persistencia de CPA-2 en diferentes superficies del *drencher*, la línea de tratamiento, las cámaras de almacenamiento y la ropa de los operarios usando PCR convencional.
b) Evaluar la dinámica poblacional de CPA-2 en la superficie de manzanas y naranjas mediante métodos moleculares y el método microbiológico tradicional.

ESTRUCTURA DE LOS ESTUDIOS REALIZADOS


Identificación y seguimiento del antagonista *P. agglomerans* CPA-2
Desarrollo de las técnicas moleculares
Evaluación de la dispersión y persistencia de CPA-2 en aplicaciones de pre y post-cosecha mediante PCR convencional
PCR cuantitativa (qPCR) *(Capítulo I)*
qPCR en combinación con el PMA (PMA-qPCR) *(Capítulo II)*
Aplicación en la cuantificación de CPA-2
En el proceso de formulación mediante liofilización, lecho fluido y atomización *(Capítulo III)*
En la superficie de la fruta en condiciones de pre *(Capítulo IV)* y postcosecha *(Capítulo I y V)*
•Hojas
•Frutos
•Suelo
•Hierbas
•Utensilios aplicación
•EPIs
(Capítulo IV)
Precosecha
Naranjas
Postcosecha
•Línea tratamiento
•Cámaras
•Envases
•EPIs
(Capítulo V)
(Capítulo I)
Manzanas
•Línea tratamiento
•Cámaras
•Envases
•EPIs

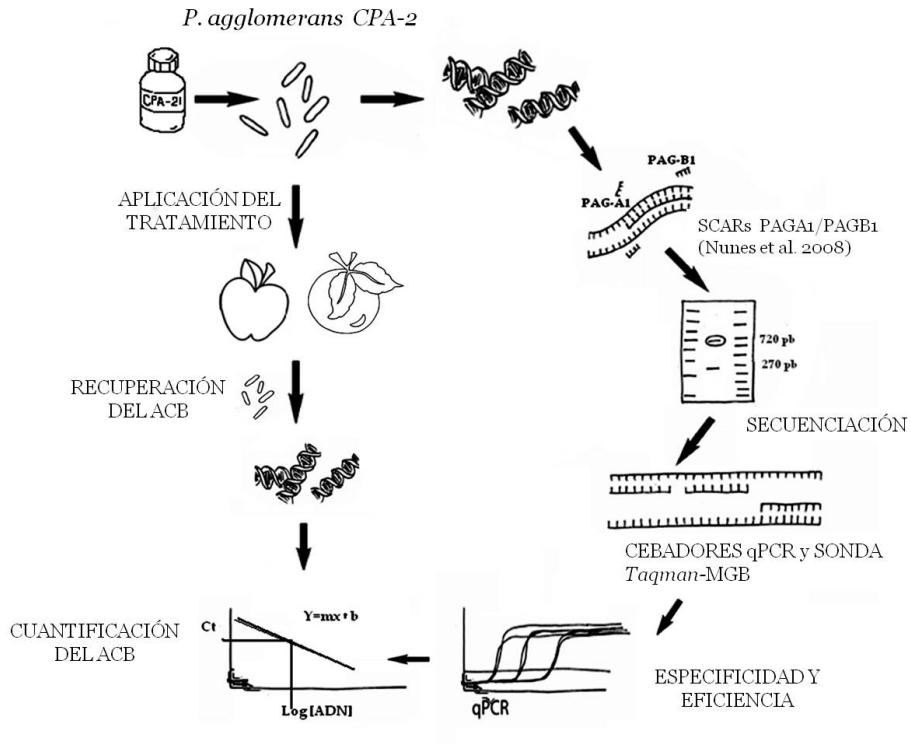
METODOLOGÍA

METODOLOGÍA

A continuación, se describe brevemente la metodología que se ha utilizado para cumplir cada uno de los objetivos de esta tesis:

1) **Desarrollo de un sistema de qPCR usando sonda *Taqman*-MGB para la cuantificación específica de *P. agglomerans* CPA-2** *(Capítulo I).*

El desarrollo de la técnica de qPCR se ha realizado partiendo de los SCARs; PAGA1 y PAGB1 previamente diseñados para *P. agglomerans* CPA-2 mediante el diseño y la optimización de unos cebadores y una sonda *Taqman*-MGB. Así mismo, la especificidad de los cebadores y la sonda fueron evaluados *in silico* y haciendo una reacción de amplificación con el ADN de diferentes cepas de *P. agglomerans* obtenidas de: *(i)* la Colección Española de Cultivos Tipo (CECT), *(ii)* la colección de microorganismos con actividad microbiológica del laboratorio de "Patologia de la Postcollita" del IRTA y *(iii)* otras cepas bacterianas aisladas de la superficie de diferentes frutos. Posteriormente, se corroboró la eficiencia de los cebadores SP_2-F/SP_2-R junto con la sonda *Taqman*-MGB. Finalmente, se obtuvo la curva de calibración con la concentración de ADN versus el *Cq* para cuantificar la concentración de los formulados de CPA-2 en cultivo puro, formulado *(Capítulo III)* y en la superficie de la fruta (*Capítulo I, IV y V).*

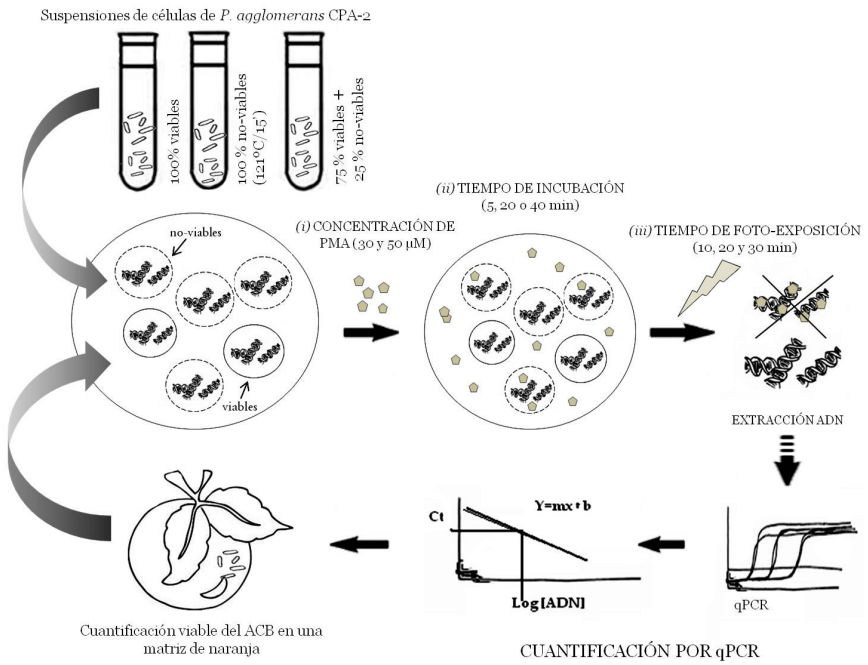


Esquema 1. Desarrollo de un sistema de qPCR usando sonda *Taqman*-MGB para la cuantificación específica de *P. agglomerans* CPA-2 *(Capítulo I)*.

2) Desarrollo de un método molecular para distinguir células viables y no viables de *P. agglomerans* CPA-2 combinando qPCR con PMA *(Capítulo II).*

Para la realización de este objetivo, se evaluaron diferentes parámetros a tener en cuenta en la puesta a punto del PMA en distintas condiciones, tales como: *(i)* la concentración de PMA (30 y 50 µM), *(ii)* el tiempo de incubación (5, 20 o 40 min) y *(iii)* el período de foto-exposición (10, 20 y 30 min) en suspensiones de células de CPA-2 100 % viables, 100 % no-viables y en una mezcla de 75 % de células viables con 25 % de células no-viables. La pérdida de viabilidad se obtuvo al someter las células de CPA-2 a una temperatura de 121 °C durante 15 min. Posteriormente, la viabilidad de CPA-2 fue evaluada mediante recuento en placa en medio NYDA e incubadas a 30 °C durante 24 h.

Por otro lado, se evaluó la toxicidad del PMA en células de *P. agglomerans* CPA-2. Una vez optimizadas las condiciones en cultivo puro, se evaluó la eficiencia de la técnica para discriminar células viables de muertas en una matriz a base del epicarpio de la naranja. Para la cuantificación por PMA-qPCR se emplearon las condiciones de PMA definidas anteriormente y se combinaron con la cuantificación mediante qPCR (*Capítulo I*).

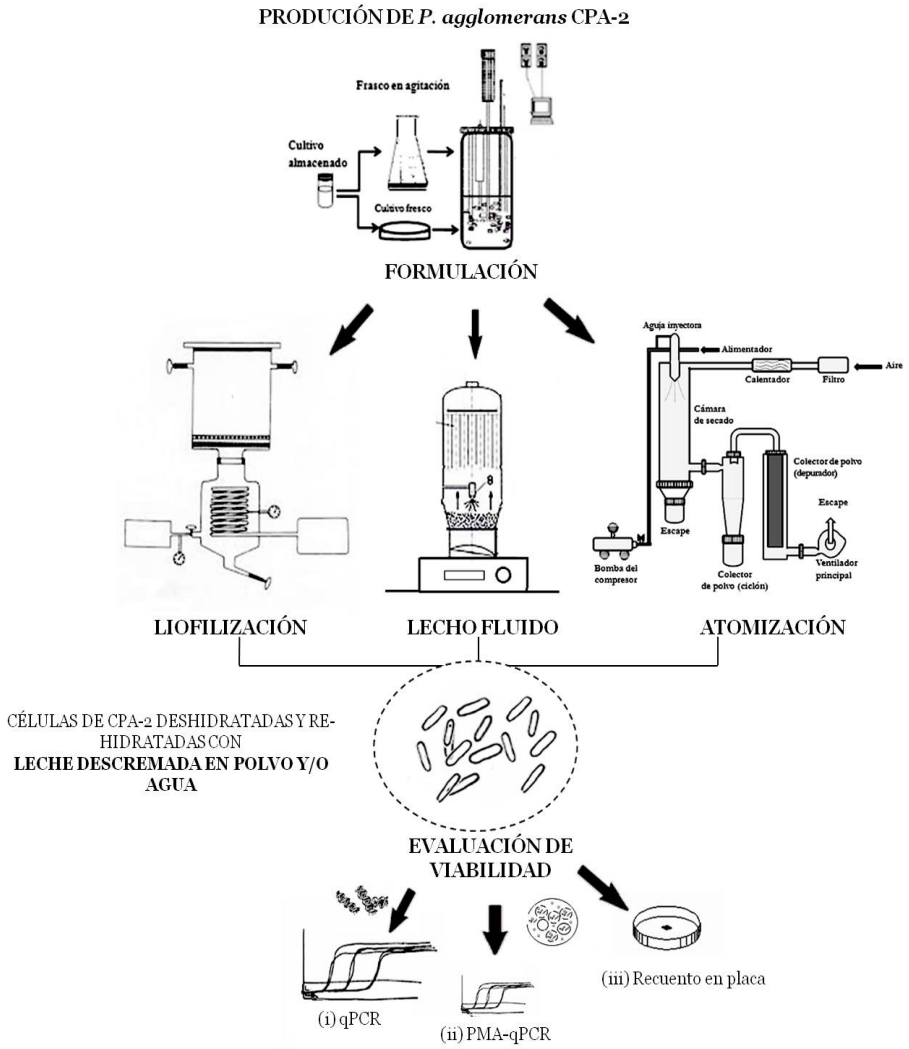


Esquema 2. Desarrollo de un método molecular para distinguir células viables y no viables de *P. agglomerans* CPA-2 combinando qPCR con PMA *(Capítulo II).*

3) Aplicación de la técnica PMA-qPCR para cuantificar células viables de *P. agglomerans* CPA-2 durante su formulación mediante liofilización, lecho fluido y atomización *(Capítulo III)*.

Para llevar a cabo este objetivo, se realizaron diferentes formulaciones secas de *P. agglomerans* CPA-2 mediante tres sistemas de secado: liofilización, lecho fluido y atomización. Para cada formulado, se evaluó la recuperación de la viabilidad usando dos rehidratantes, agua y leche descremada en polvo. La viabilidad de CPA-2 en cada uno de los formulados previamente rehidratados se evaluó mediante; *(i)* qPCR, para determinar la concentración total; *(ii)* la técnica de PMA-qPCR, para determinar la concentración de células viables y células en estado VBNC que no tengan daño en membrana y *(iii)* el recuento en placa, para determinar la concentración viable cultivable.

Finalmente, se llevó a cabo un ensayo de efectividad con las células atomizadas de *P. agglomerans* CPA-2, ajustando a dos concentraciones (dosis) del inóculo mediante el método microbiológico de recuento en placa y el molecular de PMA-qPCR, para evaluar la capacidad de control sobre *P. digitatum* en naranja cv. *Valencia Late*.



Esquema 3. Aplicación de la técnica PMA-qPCR para cuantificar células viables de *P. agglomerans* CPA-2 durante su formulación mediante liofilización, lecho fluido y atomización *(Capítulo III)* .

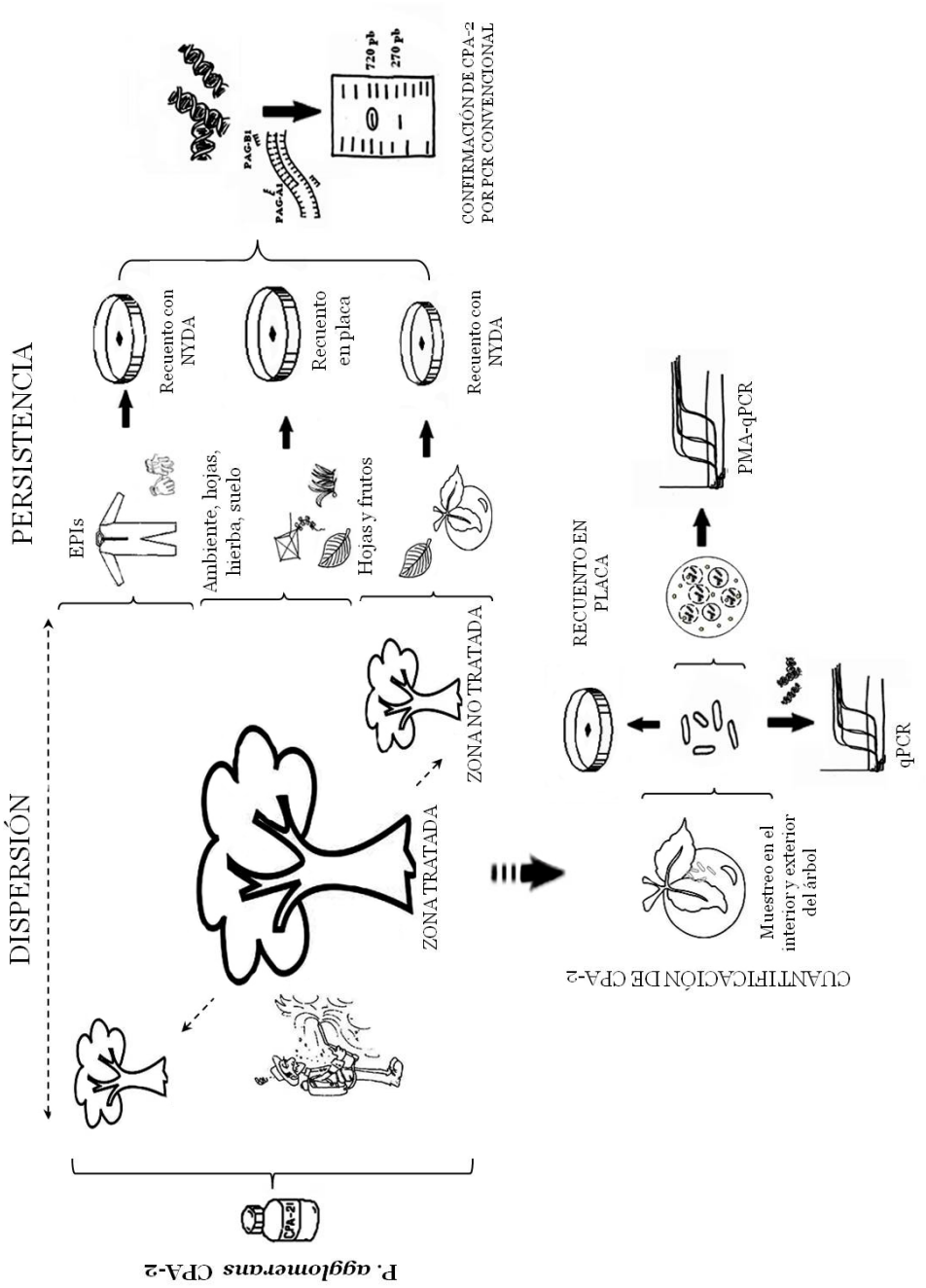
4) Estudio de la dispersión y persistencia de *P. agglomerans* en tratamiento de precosecha en naranja *(Capítulo IV).*

El estudio de persistencia y trazabilidad de *P. agglomerans* se realizó en un huerto comercial de cv. *Valencia Late* ubicado en la finca Mianes (Tortosa). Para ello, los árboles fueron tratados por pulverización con el formulado de CPA-2 en las condiciones habituales utilizadas en campo. Se trabajó en dos zonas: *(i)* zona tratada, corresponde a los árboles que fueron tratados directamente con el tratamiento de CPA-2; y *(ii)* zona no tratada, corresponde a los árboles colindantes a los de la zona tratada.

La dispersión del tratamiento se evaluó mediante papeles hidrosensibles, colocados tanto en la zona tratada como en la no tratada y en puntos intermedios entre ambas zonas. El área de cobertura de cada papel fue evaluada mediante análisis de imágenes. Por otro lado, la persistencia se evaluó muestreando el ambiente, frutos y hojas de los árboles de la zona tratada y no tratada, así como la hierba y la tierra. Además, se realizaron muestreos en la ropa de los operarios y utensilios de trabajo.

Los muestreos del ambiente en ambas zonas se realizaron por gravimetría dejando abiertas unas placas Petri con medio NYDA. Los frutos y hojas de la zona no tratada, así como los operarios y los utensilios de trabajo se muestrearon con placas Rodac con medio NYDA suplementado con imazalil. Los muestreos en hojas, hierba y tierra de la zona tratada se realizaron mediante lavado, en una solución tampón y homogeneizando en stomacher, la cuantificación de las colonias se realizó por recuento en placa. Todas las placas fueron incubadas a 30 °C durante 24 h. Finalmente, las colonias con características fenotípicas *P. agglomerans* fueron confirmadas por PCR convencional usando los marcadores SCARs específicos para CPA-2 PAGA1 y PAGB1.

En este ensayo, se cuantificó la población de CPA-2 en la superficie de naranjas recolectadas del interior y el exterior de los árboles tratados, mediante qPCR, PMA-qPCR y recuento en placa.



Esquema 4. Estudio de la dispersión y persistencia de *P. agglomerans* en tratamiento de precosecha en naranja *(Capítulo IV)*.

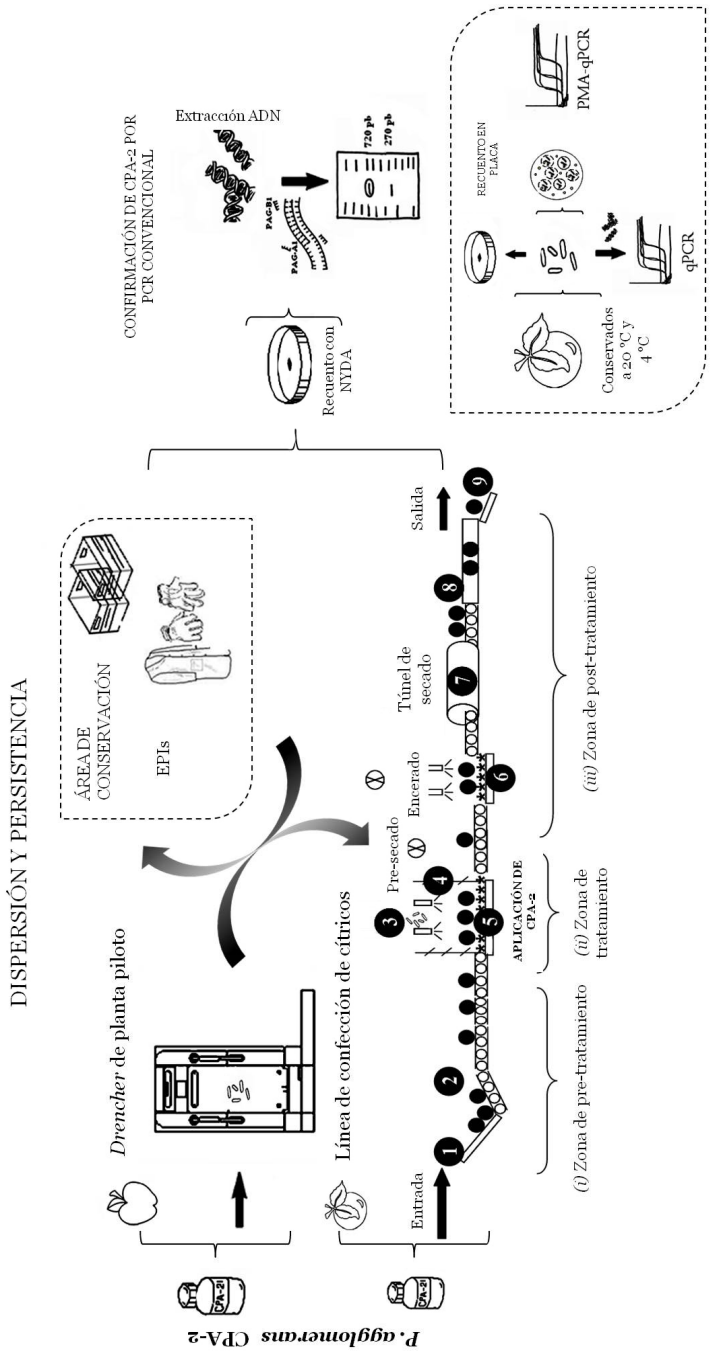
5) Evaluación de la dispersión y persistencia de *P. agglomerans* CPA-2 en tratamiento de postcosecha de manzana *(Capítulo I)* **y naranja** *(Capítulo V)***.**

Para evaluar la dispersión y persistencia del producto formulado de CPA-2 aplicado en postcosecha de fruta se llevaron a cabo dos ensayos postcosecha a escala semi-comercial: manzanas cv. *Golden Delicious* y naranjas cv. *Valencia Late*. En ambos ensayos, los muestreos en superficie se realizaron usando placas Rodac con medio NYDA suplementado con imazalil. El ambiente se muestreó por gravimetría dejando abiertas placas Petri con medio NYDA e imazalil durante dos minutos. Todas las placas fueron incubadas a 30 °C durante 24 h. Finalmente, las colonias con características fenotípicas de *P. agglomerans* fueron confirmadas por PCR convencional usando los marcadores SCARs específicos para CPA-2; PAGA1 y PAGB1.

Ensayo semi-comercial en manzana. El ensayo de postcosecha consistió en aplicar el producto formulado de CPA-2 en manzanas cv. *Golden Delicious* mediante el sistema de ducha (*drencher* de planta piloto) en las instalaciones del Centro IRTA-Lleida. Para evaluar la dispersión y persistencia de CPA-2 se muestreó el ambiente y las superficies de tres áreas diferentes: *(i)* área de tratamiento, corresponde: al ambiente en la zona de tratamiento, las superficies de la ducha y el suelo; *(ii)* área de conservación: el ambiente y superficies de las cámaras de almacenamiento (20 °C y 0 °C), así como las cajas de plástico donde se trató y guardó la fruta; y *(iii)* los equipos de protección individual (EPIs) tales como la ropa de trabajo y los guantes usados por los operarios durante el ensayo. Por otro lado, se cuantificó la población de CPA-2 en la superficie de manzanas tratadas en postcosecha y conservadas a 20 °C y 0 °C mediante qPCR y recuento en placa.

Ensayo semi-comercial en naranja. El ensayo de postcosecha consistió en aplicar el producto formulado de CPA-2 sobre naranjas cv. *Valencia Late* en la línea de confección de cítricos del centro del IRTA-Lleida. La dispersión y persistencia de CPA-2 se evaluó muestreando el ambiente y diferentes superficies de las tres áreas de muestreo: *(i)* ambiente, superficies de la línea de tratamiento y suelo, *(ii)* el área de conservación (20 °C y 4 °C): ambiente y superficies de las cámaras de almacenamiento y cajas de plástico, y *(iii)* los EPIs usados por los operarios.

La dinámica poblacional de CPA-2 de la superficie de las naranjas almacenadas a 20 °C y/o 4 °C se determinó mediante qPCR, PMA-qPCR y recuento en placa.



Esquema 5. Evaluación de la dispersión y persistencia de *P. agglomerans* CPA-2 en tratamiento de postcosecha de manzana *(Capítulo I)* y naranja *(Capítulo V)*.

CAPÍTULO I

Detection and quantification by PCR assay of the biocontrol agent *Pantoea agglomerans* CPA-2 on apples

Soto-Muñoz, L., Teixidó, N., Usall, J., Viñas, I., Torres, R.

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ABSTRACT

The registration of biological control agents requires the development of monitoring systems to detect and quantify the agent in the environment. *Pantoea agglomerans* CPA-2 is an effective biocontrol agent for postharvest diseases of citrus and pomes fruits. The monitoring of CPA-2 in postharvest semi-commercial trials was evaluated by printing Rodac plates and the colonies isolates were confirmed by conventional PCR using the SCAR primers PAGA1 and PAGB1. Samples were taken from different surfaces that had contact with CPA-2, the surrounding environment and working clothes worn by handlers. Moreover, population dynamics of the strain CPA-2 were determined on apple surfaces using both the classical plating technique and real-time PCR quantitative (qPCR). A qPCR assay using a 3'-minor groove-binding (MGB) probe was developed for the specific detection and quantification of *P. agglomerans* strain CPA-2. Based on the nucleotide sequence of the one differential fragment of CPA-2, one primer set and *Taqman* MGB probe was designed. The primers SP_2-F/SP_2-R and the *Taqman* MGB probe showed a specific detection of strain CPA-2 on apple surfaces, which was verified by BLAST analysis and tested against purified DNA from 17 strains of *P. agglomerans*, 4 related *Pantoea* species, and 21 bacterial strains from other genera isolated from whole fruit and vegetables and also fresh-cut. The detection level was approximately 10^3 cells per reaction, and the standard curve was linear within a range of 5 log units. Results from semi-commercial trials showed that CPA-2 had a low impact. The maximum persistence of *P. agglomerans* CPA-2 was 3 days in plastic boxes stored at 0 °C. Significant differences in CPA-2 population level dynamics were observed in results obtained by qPCR and dilution plating. These differences may indicate the presence of non-degraded DNA from nonviable cells. In conclusion, qPCR is a novel potential tool to quickly and specifically monitor recent surface colonization by CPA-2 populations on apple surfaces during large-scale experiments that could ensure efficient and successful treatments.

Keywords: qPCR; *Taqman* MGB probe; monitoring; biological control; postharvest; apple.

1.1. INTRODUCTION

The biological control of postharvest pathogens is an emerging technology that can overcome the main drawbacks of chemical control: fungicidal-resistant strains deregistration of fungicides and possible concerns about health and environmental impact (Droby *et al.* 2009; Viñas *et al.* 1991). The potential biocontrol activity of many bacteria and yeast as biocontrol agents (BCAs) for controlling postharvest diseases of fruit has been clearly demonstrated (Castoria *et al.* 2001; Chand-Goyal and Spotts, 1997; Ippolito *et al.* 2005; Kurtzman and Droby, 2001; Larena *et al.* 2005; Schena *et al.* 2003; Spadaro *et al.* 2004; Vero *et al.* 2013). In this context, the bacterium *Pantoea agglomerans* strain CPA-2 is an epiphytic microorganism isolated from the surface of healthy apples in Lleida (Catalonia, Spain) (Nunes *et al.* 2001) that has been evaluated in laboratory and commercial conditions (Nunes *et al.* 2002; Torres *et al.* 2007) and has defined modes of action (Poppe *et al.* 2003), production (Costa *et al.* 2001) and formulation (Costa *et al.* 2000; 2002). CPA-2 has been optimised for preharvest applications (Cañamás *et al.* 2008a; 2009) and has been integrated with other alternative methods (Plaza *et al.* 2004; Teixidó *et al.* 2001; Usall *et al.* 2008). Other studies have described ways to increase its range of activity (Cañamás *et al.* 2008a) and defined its packaging and storage conditions.

The registration of a biocontrol product is required before any commercial use, as a usual food safety procedure. The registration of biocontrol products for postharvest use in the USA (through the EPA) has been straightforward, and several products have received registration. In Europe, however, the situation is more complex, and until recently, registration has been difficult or impossible to obtain (Droby *et al.* 2009; Fravel, 2005). The registration for placing plant protection products on the market was regulated by Council Directive 91/414/EEC and was amended by Directives 2001/36/EC and 2005/25/EC to adapt to special requirements for plant protection products based on microorganisms. In 2009, the new EC regulation 1107/2009 replaced Directive 91/414/EEC and defined time frames for the registration process. In particular, Annex II of the directive established issues that need to be addressed, including the estimation of the fate and distribution of the microorganism in the environment and its impact on non-target species. In addition to the information generated for registration, monitoring the environmental presence of a BCA is crucially important to understand its

interaction(s) with its environment and for ecological and safety assessments (Larena and Melgarejo, 2009).

Several monitoring methods have been developed to identify and quantify microorganisms. The choice of method used depends on the required level of specificity. A strain-specific detection level of BCAs is needed because they are released into the environment where other strains of the same species of the biocontrol agent may be present (El Hamouchi *et al.* 2008). Among microbiological monitoring methods, the dilution plating method has been successfully used (De Cal *et al.* 2009; Janisiewicz and Jeffers, 1997; Teixidó *et al.* 1999) and has contributed to a better understanding of the dynamics of microorganisms. However, this method does not permit a distinction between introduced and indigenous strains (Schena *et al.* 2000).

The use of molecular markers to distinguish BCA strains of other epiphytic microorganisms present on the surfaces of fruit offers much promise for the rapid identification of this specific bacterial strain. A polymerase chain reaction (PCR) method has been designed using genotypic markers such as sequence-characterised amplified regions (SCARs) for the specific identification of *P. agglomerans* strain CPA-2 (Nunes *et al.* 2008). PCR is a sensitive technology that offers several advantages over dilution plating: the technique possesses the potential to detect a single target molecule in a complex mixture, and it is rapid, versatile and, depending on the design of primers, enables strain-level detection (Schena *et al.* 2004). However, by conventional PCR, accurate quantification of DNA is not possible. This shortcoming has been overcome by the emergence of new techniques that can quantify nucleic acids in vitro. Real-time quantitative PCR (qPCR) is one of these new techniques. This technique can be performed using different fluorometric detection systems, such as the DNA-binding dye SYBR Green I or the *Taqman* hydrolysis probe. SYBR Green I dye has been utilised for the quantification of BCAs (Larena and Melgarejo, 2009; Spotts *et al.* 2009), but a disadvantage of this dye is their lack of specificity. As a consequence, a dissociation-curve is necessary to ensure that all captured fluorescence is to the amplification of interest (Schena *et al.* 2013). Unlike SYBR Green, the *Taqman* hydrolysis probe tends to be more specific due to the use of the sequence-specific probe. BCAs have also been monitored with the *Taqman* probe, although to a more limited extent. For instance, Pujol *et al.* (2006) used the *Taqman* probe to assess the environmental fate of *Pseudomonas fluorescens* EPS62e at the strain level once it was introduced into the apple phyllosphere. In addition, a more sophisticated *Taqman* probe, called 3'-minor groove-binder-DNA (MGB) probes, which forms extremely stable duplexes with the target DNA, has been used for the specific quantification of *Candida oleophila* strain O on apple surfaces under postharvest conditions (Massart *et al.* 2005). Moreover, qPCR assay was developed for specific quantification of biocontrol *P. agglomerans* strain E325, an effective BCA

against fire blight on pome fruits, with objective to provide a tool that will facilitate monitoring of the environmental behaviour of this BCA (Braun-Kiewnick *et al.* 2012).

The main objectives of the present study were: *(i)* to develop a qPCR method for the specific detection and quantification of *P. agglomerans* strain CPA-2 on apple surfaces after postharvest application and *(ii)* to study the persistence of CPA-2 applied in semi-commercial conditions by conventional PCR using SCAR primers PAGA1 and PAGB1.

1.2. MATERIAL AND METHODS

1.2.1. Fruit

Apples (*Malus domestica*) cv *Golden Delicious* were used in all experiments. Fruits were obtained immediately after harvest from commercial orchards in Lleida, Catalonia (Spain) and selected by hand for uniformity of size and ripeness. The fruits were stored without any chemical postharvest treatments at 1 °C and 85 % relative humidity (RH) before use. Fruits were used after less than one month of storage at 1 °C.

1.2.2. Antagonist

The CPA-2 strain of *P. agglomerans* used in this study was obtained from IRTA Centre in Lleida (Catalonia, Spain). This strain was isolated from *Golden Delicious* apples and is currently deposited at the Spanish Collection of Type Culture (CECT, University of Valencia, Valencia, Spain), as CECT-4920. Stock cultures were stored long-term at -80 °C in glass beads and subcultured on nutrient yeast dextrose agar (NYDA: 8 g/L nutrient broth, 5 g/L yeast extract, 10 g/L dextrose and 20 g/L agar). The activated culture was maintained on NYDA at 30 °C for 24 h and transferred to potassium phosphate buffer (pH 6.5) (0.2 M KH_2PO_4, 70 mL; 0.2 M K_2HPO_4, 30 mL and deionised water, 300 mL) to obtain a cell suspension. This suspension was used as an inoculum for biomass production in a fermentation system. Osmotically adapted cells grown in basal liquid medium (5 g/L yeast extract, 10 g/L sucrose and 25 g/L NaCl), as previously described Cañamás *et al.* (2008b), were used in all studies. An appropriate volume of inoculum was added to 5 L of the basal liquid medium adjusted to 1×10^6 CFU/mL. Cultures were grown in a 5 L bench-top BIOSTAT-A fermenter (Braun Biotech International, Melsungen, Germany) at 30 °C with 300 rpm agitation and 100 L/h aeration. Cultures were harvested at stationary phase (24 h) by centrifugation at 9820 *g* for 10 min at 15 °C in an AvantiTM J-25 centrifuge (Beckman, Palo Alto, Ca, USA). Cell pellets were resuspended in deionised water containing 10 % sucrose, frozen

at -20 °C overnight and freeze-dried (Cryodos, Telstar SA, Terrasa, Catalonia, Spain) at 1 Pa and -45 °C for 24 h as described by Costa *et al.* (2000). These lyophilised cells were packaged in a white high-density polyethylene bottle at non-vacuum atmosphere (Torres *et al.* unpublished data). For the postharvest application of the biocontrol agent, cell pellets were resuspended in 1 % non-fat skim milk (Sveltesse, Nestle, Vevey, Switzerland) and used immediately to treat apples.

1.2.3. DNA extraction

Genomic DNA was extracted using two DNA extraction methods. The first method was used during processing of samples for conventional PCR monitoring; it is a rapid method of DNA extraction based on the Lyse-N-Go PCR Reagent (Pierce Chemicals, Rockford, IL) and can be applied on a single colony. The second method was used for the qPCR for quantification assay. The protocol was as follows: 500 µl of formulated and rehydrated cells adjusted to 10^7 CFU/mL was centrifuged at 9727 *g* for 20 min (Hettich Mikro 22R, Germany). The pellet was resuspended in 200 µl of breaking buffer (200 mM Tris HCl pH 7.5, 250 mM NaCl, 25 mM EDTA, 0.5 % SDS, 2 % PVP). This cell suspension was vortexed and vigorously shaken in a FastPrep machine (FP120, Bio101, Savant) at speed position 4.0 for 30 s twice in the presence of 0.3 g of acid-washed glass beads (425–600 µm diameter) and 200 µl of chloroform/isoamyl alcohol (24:1). The sample was then centrifuged at 4766 g for 5 min at 4 °C after the addition of 200 µl TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The aqueous phase was transferred into a new microcentrifuge tube and incubated at 37 °C for 15 min after the addition of 1 µg/µL RNase A (RPA grade enzymes, Ambion). An equal volume of isopropanol was added, and the tube was gently inverted several times to precipitate the DNA and then was placed at -20 °C for 30 min. The mixture was centrifuged at 12,863 *g* for 10 min at 4 °C, and the supernatant was discarded. The precipitated DNA was washed with 70 % ethanol and dried briefly. The dried DNA pellet was resuspended in 20 µL of sterile ultrapure water. The solution was stored at -20 °C until its use. The amount and purity of DNA samples were determined spectrophotometrically (NanoDrop ND-1000; NanoDrop Technologies, Wilmington, DE), and DNA integrity was analysed by electrophoresis on 1.5 % agarose gels run at 70 V for 90 min with TEB buffer and stained with 0.5 mg/µL ethidium bromide (Sambrook and Russell, 2001).

1.2.4. Development of qPCR for CPA-2 quantification

The primers pair PAGA1 (ATGAGCCCTGTGATCAGGAAGATCG) and PAGB1 (ACGATGAGCCTTCTCAGCAAATGCG) designed for *P. agglomerans* CPA-2 by (Nunes *et al.* 2008) amplify two different fragments. A first SCAR marker (720 bp) is specific to strain CPA-2 and the second one (270 bp) may be specific to *P. agglomerans* species. The two SCARs marker were purified using the minElute Gel Extraction kit (Qiagen,

USA) and then sequenced using the sequencing services of Ez-Seq Magcrogen (Amsterdam, Holland). The nucleotide sequences of both products, 720 and 270 bp, were aligned to identify the variable region using the software MEGA program version 3.1 (http://www.megasoftware.net/).

One primer set (SP_2-F CTTAAAGCGCAGGGAAGCCGGTCAG and SP_2-R GAGCCGGCTCAGGGAAACCGGTC) and a *Taqman*-MGB probe (FAM-TCCATGGATGGCTTAAG-MGB) were designed to amplify a fragment of approximately 100 bp using Primer ExpressTM version 2.0 software (PE Applied Biosystem Division, Perkin-Elmer Co. Foster City, CA, USA). The primer set and *Taqman* MGB probe was designed within the sequence between primers following the criteria of Kutyavin *et al.* (2000). The *Taqman* MGB probe was supplied by Applied Biosystems (Barcelona, Spain) with a 5' covalently attached 6-carboxifluorescine (FAM) reporter dye, a nonfluorescent quencher and an MGB moiety at the 3' end.

The ABI-7500 qPCR detection system (Applied Biosystem Division, Perkin-Elmer Co. Foster City, CA, USA) was used for qPCR development to detect and quantify genomic CPA-2 DNA. Each qPCR was performed in MicroAmp optical 96-well plates that were sealed with an optical adhesive cover (Applied Biosystem, Foster City, CA, USA). The concentrations of the primers and the probe were optimised, and the qPCR was performed in a reaction volume of 10 µL. The reaction mixture contained 1× PCR *Taqman* Universal PCR Master Mix, 300 nM of each primer, 200 nM of *Taqman* MGB probe and 2 µL of extracted DNA. Cycling conditions were: 2 min at 50 °C, 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 59 °C. All reactions were performed in triplicate. Data acquisition and analysis were performed using SDS software version 1.3.1 (Applied Biosystems) according to the manufacturer's instructions. The quantification cycles (*Cq*) necessary to generate a fluorescent signal significantly above the noise level were automatically calculated for each reaction by the SDS software. Any sample that reached a fluorescence value exceeding the fluorescence threshold value was considered positive.

Assessment of primer and *Taqman* MGB probe specificity was evaluated by applying two different approaches: *(i)* by BLAST (Basic Local Alignment Search Tool) analysis, to explore the available DNA sequences in the NCBI GenBank database (www.ncbi.nlm.nih.gov/BLAST/) and exclude the presence of matching sequences in other microorganisms and *(ii)* by testing primers and probe against genomic DNA from 17 strains of *P. agglomerans*, 4 related *Pantoea* species, and 21 bacterial strains from other genera isolated from whole fruit and vegetables and also fresh-cut (Table 2). Bacteria were grown on NYDA medium at 30 °C for 24 h. For DNA extraction from pure cultures, bacterial colonies were resuspended in 500 µL of potassium phosphate buffer

(pH 6.5) and the cell suspensions were processed according to the second method of DNA extraction described in Section 1.2.3. Finally, 10 ng of DNA per PCR reaction was analysed by qPCR under conditions mentioned above, a negative control without DNA and a positive control of CPA-2 were included. All reactions were performed in triplicate.

Absolute quantification of *P. agglomerans* CPA-2 was achieved by constructing a standard curve. A standard quantification curve was prepared by adding a range of bacterial cell concentrations (from 2×10^7 to 2×10^2 CFU/mL) to apple suspensions. These apple suspensions were obtained from 40 pieces of peel (14 mm diameter) of five untreated fruits in 15 mL of 0.05 M phosphate buffer solution, pummelled in a Stomacher 400 (Seward, London, UK) set at normal speed for 90 s. DNA extraction was done as described in Section 1.2.3. A negative control without CPA-2 was included. All reactions were performed in triplicate. The *Cq* value that determines the cycle number at witch fluorescence increases above background, was plotted against the logarithm of starting quantity of template for each dilution. Amplification efficiency was calculated from slope of the standard curve $E=10^{-1/slope}$; % Efficiency = (E-1) x 100.

1.2.5. Semi-commercial trial using *P. agglomerans* CPA-2

A semi-commercial trial using a pilot drencher was conducted at IRTA-Centre in Lleida. The treatment was conducted on November 15th, 2011, at ambient temperature (approximately 12 °C). Apples were placed in ten plastic boxes containing 60 fruits each. Fruit boxes were drenched with a formulated and packaged product of *P. agglomerans* CPA-2 adjusted to a concentration of 2 x 10^7 CFU/mL following standard industrial procedures. Fruit in untreated boxes were used as controls. After treatment, fruit boxes were separated into two sets: one set was stored at 20 °C and 80 % RH for 9 days, and the other set was stored at 1 °C and 85 % RH for 68 days.

1.2.6. Study of the dispersion and persistence of *P. agglomerans* CPA-2 in postharvest applications

Three sample types were selected to monitor CPA-2 on different surfaces and environments: *(i)* treatment area, *(ii)* storage chambers (at 20 °C and 0 °C) and *(iii)* gloves and working clothes worn by handlers.

The air of each zone was sampled according to the gravimetric method. Three petri dishes (replicates) containing NYDA medium supplemented with imazalil (imazalil sulphate 99 %, 0.5 g/L) were equidistantly distributed through each zone and left open for 2 min. All surfaces were sampled with Rodac (replicate organism direct agar contact) plates containing NYDA medium with 0.5 g/L imazalil by contact between the culture medium and the surface, with slight pressure to allow adhered microorganisms to reach the medium.

The sample points in the treatment area were: *(i)* the drencher surfaces before and after treatment, after cleaning and rinsing and 1 and 2 days later and *(ii)* the floor and environment during the assay (day 0) and 1 day after. For each sampling, six Rodac plates (replicates) were used both in the drencher and on the floor. In storage chambers (at 20 °C and 0 °C), the following locations were sampled: *(i)* the floor, walls and the air within each storage chamber just after the treatment (day 0) and 1 day after treatment and *(ii)* the surface inside and outside of two plastic boxes located in each storage chamber at days 0, 1, 3, 5 and 6 after treatment. For each sampling, four Rodac plates were used in each storage chamber surface (floor and walls) and each plastic box. Finally, the gloves and working clothes worn by two handlers during the semi-commercial assay were sampled at day 0 and 1 and 2 days after treatment. For each sampling, two Rodac plates were used on each glove pair, and four Rodac plates were used on the working clothes of each handler.

The Rodac and NYDA plates used for the different samplings were incubated at 30 °C for 48 h. To distinguish *P. agglomerans* CPA-2 from other microorganisms, yellow colonies with phenotypic characteristics similar to *P. agglomerans* were randomly selected from plates of all sampling days. The number of selected colonies was at most 25 colonies per plate but was always at least 40 % of all colonies per plate.

The selected colonies were analysed and identified by conventional PCR using the primers pair PAGA1 (ATGAGCCCTGTGATCAGGAAGATCG) and PAGB1 (ACGATGAGCCTTCTCAGCAAATGCG) designed for *P. agglomerans* CPA-2 (Nunes *et al.* 2008). Fifty microliters of amplification reaction were prepared using 20 ng of each genomic DNA, 0.5 units of Taq DNA polymerase in 1 × reaction buffer (Invitrogen Life Technologies, Carlsbad, CA, USA), 0.2 mM dNTPs and 0.4 mM of each SCAR primer. DNA amplification was carried out in a peltier thermal cycler (GeneAmp PCR System 2700, Applied Biosystems) with the following program: an initial denaturation step at 95 °C for 5 min followed by 40 cycles of 95 °C for 30 s, 59 °C for 45 s and elongation at 72 °C for 90 s followed by a final extension step at 72 °C for 10 min. The PCR products were separated by electrophoresis on 1.5 % agarose gel as described in Section 1.2.3. DNA standard (100 bp DNA Ladder, Invitrogen Life Technologies, Carlsbad, CA, USA) was used as a molecular size marker. Experiment was performed twice.

1.2.6.1. Quantification of P. agglomerans *on apple surfaces*

The quantification of *P. agglomerans* CPA-2 on apples stored at 20 °C and 0 °C was carried out by two methods: dilution plating and qPCR. The recovery of CPA-2 cells from apple surfaces was performed according to the method described by Torres *et al.* (2012), with minor modifications. Briefly, eight pieces of peel were removed from each fruit using a cork borer with a diameter of 14 mm. Peel samples were mixed with 15 mL of

0.05 M phosphate buffer solution in a sterile Stomacher filter bag and pummelled (stomached) in a Stomacher 400 set at normal speed for 90 s. For quantification by dilution plating, ten-fold dilutions were made in phosphate buffer solution and plated on NYDA media. Each suspension was analysed twice by dilution plating. The colonies were counted after incubation at 30 °C for 24 h. Five fruits constituted a single replicate, and four replicates were performed. For analysis by qPCR, genomic DNA were extracted from 2 mL of each sample as described in Section 1.2.3, and was diluted 10-fold prior to qPCR. Each DNA extraction was evaluated using *Taqman* MGB probe designed specifically for CPA-2. The amount of resulting CPA-2 cells was calculated according to the generated standard curve. DNA that was extracted from untreated fruit was used a negative control. All reactions were performed in triplicate.

1.2.7. Statistical analysis

The *P. agglomerans* CPA-2 population level from both methods was calculated as CFU or cells/cm^2 of apple surface with its standard error and a 95 % confidence interval. Population size (CFU/cm^2) was log transformed to improve homogeneity.

1.3. RESULTS

1.3.1. Development of a qPCR assay for CPA-2 DNA

Sequence analysis of both the 720 and 270 bp products generated with the primers PAGA1 and PAGB1 showed a variable region located between positions 547 and 695 of the 720 bp fragment. At 10 ng DNA per reaction (approximately 10^6 cells per reaction), successful amplification of CPA-2 was achieved (*Cq* values from 24 to 25) for SP_2-R/SP_2-F primers and *Taqman* MGB probe designed from the variable region identified for CPA-2. The specificity was proven because under these conditions, no fluorescence was detected for any of the strains tested (Table 1).

Using the standard curve with the SP_2-R/SP_2-F primers, based on dilutions of CPA-2 cells mixed with an apple suspension, a good linearity was observed over a 4-log range (y=45.09-3.152x) (Figure 1). The limit of detection was approximately 10^3 cells per reaction with an average *Cq* value of 35.04 (corresponding 4.9×10^2 cells/cm^2 of apple surface).

1.3.2. Study of dispersion and persistence of *P. agglomerans* CPA-2 in postharvest application

The total number of colonies of *P. agglomerans* CPA-2 isolated from the sampled treatment areas is shown in Table 2. The sample of drencher before treatment did not show the presence of strain CPA-2 ; in contrast, a high number of CPA-2 colonies was detected immediately after treatment (>10^3 CFU/Rodac plate).

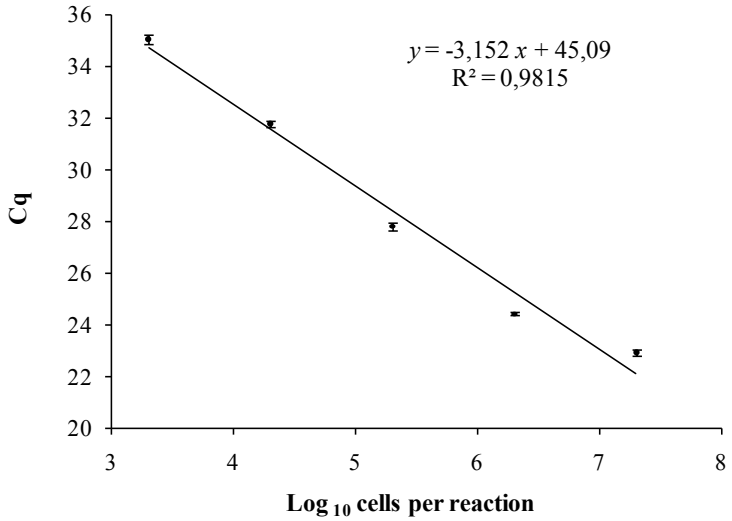

Figure 1. Standard curve obtained with *Taqman* assay based on dilutions of *P. agglomerans* (CECT 4920) cells mixed with an apple suspension. The concentrations assayed were: from 2×10^7 to 2×10^3 cells/mL. The figure shows a strong linearity between *Cq* values and the concentration of *P. agglomerans* cells assayed (R2 = 0.98) and an efficiency of 107 %.

Table 1. Results of the qPCR specificity assays for *P. agglomerans* and other bacterial species tested with SP_2-F/SP_2-R primers and *Taqman* MGB probe designed for quantitative PCR detection of *P. agglomerans* CPA-2.

Genus/species	Strain	Source of isolation	qPCR Result
Target			
Pantoea agglomerans	CPA-2	Apple	+[a]
Pantoea agglomerans	CECT 4842	Industrial	- [b]
Pantoea agglomerans	CECT 850	Human	-
Pantoea agglomerans	OC-192	Environment	-
Pantoea agglomerans	OC-303	Citrus orchard (leaf)	-
Pantoea agglomerans	OC-200	Citrus orchard (environment)	-
Pantoea agglomerans	OC-227	Citrus orchard (weed)	-
Pantoea agglomerans	OC-216	Citrus orchard (environment)	-
Pantoea agglomerans	OC-213	Citrus orchard (leaf)	-
Pantoea agglomerans	OC-286	Citrus orchard (environment)	-
Pantoea agglomerans	OC-54a	Citrus orchard (weed)	-
Pantoea agglomerans	OC-245	Citrus orchard (fruit)	-
Pantoea agglomerans	OC-231	Citrus orchard (leaf)	-
Pantoea agglomerans	OC-217	Citrus orchard (fruit)	-
Pantoea agglomerans	BP1-25	Peach	-
Pantoea agglomerans	BP1-14	Peach	-
Pantoea agglomerans	BP1-27	Peach	-
Pantoea agglomerans	-	Human	-
No target			
Bacillus amyloliquefaciens	188/03	Stone fruit	-
Bacillus amyloliquefaciens	62/03	Stone fruit	-
Bacillus amyloliquefaciens	58/04	Stone fruit	-
Bacillus amyloliquefaciens	B12	Stone fruit	-
Bacillus atrophaeus	24/04	Stone fruit	-
Bacillus mojavensis	100/03	Stone fruit	-
Bacillus mojavensis	28/03-A	Stone fruit	-
Bacillus sp.	118/03	Stone fruit	-
Bacillus sp.	C-5 10	Pome fruit	-
Bacillus sp.	P12	Pome fruit	-
Flavimonas oryzihabitans	PN5	Apple	-
Hafnia alvei	M201 Psi3 E6	Lettuce whole	-
Lysinibacillus sphaericus	F-13	Pome fruit (leaf)	-
Pantoea ananatis	CPA-3	Apple	-
Pantoea sp.	M247	Lettuce	-
Pantoea sp.	128-M	Apple	-
Pantoea sp.	EL8	Peach	-
Pseudomonas graminis	CPA-7	Apple	-
Pseudomonas sp.	M309 Psi7 E7	Ook lettuce	-
Pseudomonas syringae	CPA-5	Apple	-
Pseudomonas syringae	F-10	Pome fruit (leaf)	-
Pseudomonas trivialis	M230 Psi2 E3	Iceberg lettuce (fresh-cut)	-
Rahnella aquatilis	RG4	Peach	-
Rahnella sp.	M172 Psi1 PO2	Apple (fresh-cut)	-
Serratia sp.	M290 Psi4 E4	Iceberg lettuce	-

[a] (+) indicates a weak positive fluorescence signal at lower *Cq* values (<38).

[b] (-) indicates no fluorescence increase detected after 38 PCR cycles.

Cleaning and rinsing of the drencher with water were not sufficient to completely remove CPA-2 ; however, levels of CPA-2 decreased to less 10 CFU/Rodac plate. After the sampling on day 1 only 2 of 6 Rodac plates contained more than 1 colony with phenotypic characteristics similar to *P. agglomerans,* but conventional PCR of their DNA did not produce the 720 bp amplified fragment specific for CPA-2. Floor samples showed that CPA-2 was detected only immediately after treatment. Finally, no CPA-2 was detected at any time in the air. The presence of *P. agglomerans* CPA-2 in the environment and on different storage chamber surfaces (at 20 °C and 0 °C) such as floors and walls was not detectable (Table 3). No colony identified as the CPA-2 strain was detected by conventional PCR on the walls of the storage chamber at 0 °C. In plastic boxes, the CPA-2 strain was detected from the first samplings: the initial concentration was more than 10^3 CFU/Rodac plate on plastic boxes at both 20 °C and 0 °C. At sampling day 1 in plastic boxes at 20 °C, 2 of 8 Rodac plates presented more than 10^2 CFU/Rodac plate, and all colonies selected were identified as CPA-2 by conventional PCR. After sampling day 3, only 1 of 8 Rodac plates presented more than 1 colony with phenotypic characteristics similar to *P. agglomerans,* but neither colony was identified as CPA-2.

Table 2. Total number of colonies isolated from different treatment areas with appearances similar to *P. agglomerans* that were identified by conventional PCR to be CPA-2.

Sample	Sampling time (days)	Rodac plate	CFU/Rodac	CPA-2 confirmed by PCR (%)
Drencher	Before treatment	0/6[a]	-	-
	After treatment	6/6	>10^3	100[b]
	After cleaning with water	4/6	>10	100
	1	2/6	>1	0
	2	0/6	-	-
Floor	0	2/6	>10^2	100
	1	0/6	-	-
Air	0	0/3	-	-
	1	0/3	-	-

[a] Total number of Rodac plates that showed the presence of colonies with appearances similar to *P. agglomerans* in relation to total number of plates.

[b] Percentage of colonies confirmed to be the CPA-2 strain by conventional PCR. The number of colonies selected was at most 25 colonies per plate, but always at least 40 % of all colonies per plate were analysed by conventional PCR.

Table 3. Total number of colonies isolatedfrom storage chambers with appearances similar to *P. agglomerans* that were identified by conventional PCR to be CPA-2.

Sample	Storage chamber	Sampling time(days)	Rodac plate	CFU/Rodac	CPA-2 confirmed by PCR (%)
Air	20 °C	0	0/3[a]	-	-
		1	0/3	-	-
	0 °C	0	0/3	-	-
		1	0/3	-	-
Floor	20 °C	0	0/4	-	-
		1	0/4	-	-
	0 °C	0	0/4	-	-
		1	0/4	-	-
Walls	20 °C	0	0/4	-	-
		1	0/4	-	-
	0 °C	0	2/4	>10	0[b]
		1	0/4	-	-
Plastic Boxes	20 °C	0	6/8	$>10^3$	100
		1	2/8	$>10^2$	100
		3	1/8	>1	0
		5	0/8	-	-
	0 °C	0	2/8	$>10^3$	100
		1	4/8	$>10^2$	82
		3	3/8	>10	100
		5	2/8	>1	0
		6	0/8	-	-

[a] Total number of Rodac plates that showed the presence of colonies with appearances similar to *P. agglomerans* in relation to total number of plates.

[b] Percentage of colonies confirmed to be the CPA-2 strain by conventional PCR. The number of colonies selected was at most 25 colonies per plate, but always at least 40 % of all colonies per plate were analysed by conventional PCR.

Consequently, the persistence of CPA-2 was not longer than 3 days in the plastic boxes stored at 20 °C. At sampling day 1 for the plastic boxes maintained at 0 °C, 4 of 8 Rodac plates presented more than 10^2 CFU/Rodac plate, and 82 % were identified as the CPA-2 strain. At day 3, the presence of similar colonies was still detected, and all of them were identified as CPA-2. At sampling day 5, 2 of 8 Rodac plates presented more than 1 colony with phenotypic characteristics similar to *P. agglomerans*, but neither of them was identified as CPA-2 by conventional PCR. Finally, at sampling day 6, CPA-2 was not detected on Rodac plates.

Table 4. Total number of colonies isolated from handlers' clothes with appearances similar to *P. agglomerans* identified by conventional PCR to be CPA-2.

Sample	Sampling time (days)	Rodac plate	CFU/Rodac	CPA-2 confirmed by PCR (%)
Gloves	0	4/4[a]	$>10^3$	87.5[b]
	1	0/4	-	-
Working clothes	0	8/8	$>10^3$	100
	1	5/8	>10	80
	2	0/8	-	-

[a] Total number of Rodac plates that showed the presence of colonies with appearances similar to *P. agglomerans* in relation to total number of plates.

[b] Percentage of colonies confirmed to be the CPA-2 strain by conventional PCR. The number of colonies selected was at most 25 colonies per plate, but always at least 40 % of all colonies per plate were analysed by conventional PCR.

Results of sampling conducted on the gloves and working clothes worn by handlers are shown in Table 4. The gloves worn by handlers at sampling day 0 produced colonies on 4 of 4 Rodac plates, with more than 10^3 CFU/Rodac plate; however, only 87.5 % of colonies selected were identified as the CPA-2 strain. The persistence of the BCA on the gloves was less than 1 day, because the CPA-2 strain was not detected after this time. In addition, on working clothes, the CPA-2 strain was isolated and confirmed at day 0. At sampling day 1, 5 of 8 Rodac plate presented more than 10 CFU/Rodac plate, and 80 % of colonies selected were identified as the CPA-2 strain by conventional PCR. Finally, at day 2, no colonies with morphology similar to CPA-2 were found. The persistence of the CPA-2 strain on working clothes was less than 2 days.

1.3.2.1. Quantification of P. agglomerans *on apple surfaces*

The quantification of *P. agglomerans* CPA-2 on apples at two different storage conditions of 20 °C and 0 °C was evaluated by two methods, dilution plating and qPCR (Figure 2). The population levels of the CPA-2 strain recovered from the surface of apples stored at 20 °C are shown in Figure 2A. The amount of *P. agglomerans* CPA-2 quantified by dilution plating was approximately 3.00 $\log_{10}$ CFU/cm^2 at time 0 and decreased progressively to 0.86 $\log_{10}$ CFU/cm^2 at the end of the assay (9 days). By qPCR, the initial population levels were 3.30 $\log_{10}$ cells/cm^2 and remained quite stable, with a population of approximately 3.46 $\log_{10}$ cells/cm^2 at the end of the assay. At short time points (0-3 days), no differences were found between the two methods; however, population levels estimated by PCR at the end of the assay (6-9 days) were approximately 2.60 $\log_{10}$ cells/cm^2 higher than those assessed by the dilution plating method.

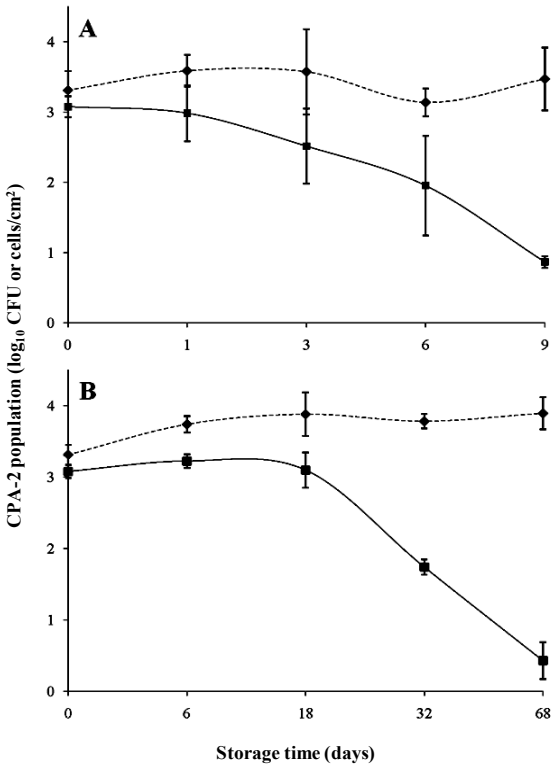

Figure 2. Population dynamics estimated by qPCR (♦) and plating (■) of *Pantoea agglomerans* CPA-2 on the apple surface. Fruits were treated with the agent and stored at 20 °C (A) or under cold storage condition at 0 °C (B). The points represent the means of four biological replicates, and the vertical bars indicate the standard deviation of the mean.

Figure 2B shows the population levels of CPA-2 recovered from the surfaces of apples stored at 0 °C. The population of CPA-2 quantified by dilution plating on apples stored at 0 °C started at approximately 3.00 $\log_{10}$ CFU/cm² and remained constant during the first 18 days of storage (3.70 $\log_{10}$ CFU/cm²). Beginning at day 18 of storage, the CPA-2 populations decreased progressively until they reached 0.42 $\log_{10}$ CFU/cm² at the end of the assay (68 days of storage). By qPCR, the population levels began at 3.30 $\log_{10}$ cells/cm² and slightly increased to 3.73 $\log_{10}$ cells/cm² at day 18. After day 18, population levels remained constant until the end of the assay (68 days). In general, the results showed differences between the methods used to estimate the population levels after the first 6 days of storage. At the end of the assay, there were differences between qPCR and dilution plating methods; the population of *P. agglomerans* CPA-2 declined approximately 3.47 $\log_{10}$ CFU or cells/cm².

1.4. DISCUSSION

The implementation of a BCA to control postharvest pathogens involves different phases, one of which is the development of a monitoring method that assures its specific detection and quantification after postharvest application. To guarantee its efficacy, the BCA must be able to colonise and survive in the target organ. Therefore, the knowledge of the environmental fate of the formulated and packaged product based on *P. agglomerans* CPA-2 under postharvest application can generate valuable data for registration purposes.

In this work, we have validated a qPCR assay using a *Taqman* MGB probe for *P. agglomerans* CPA-2 strain-specific detection. This qPCR method was used to quantify *P. agglomerans* CPA-2 on apple surfaces after postharvest application. In addition, the presence and persistence of BCA in the environment and packinghouse facilities was also monitored in a semi-commercial trial by conventional dilution plate method and identify cultured colonies by conventional PCR using the SCAR primers PAGA1 and PAGB1.

The use of BCAs under commercial conditions creates a need to distinguish the particular strain applied from indigenous microbiota. Molecular methods have been developed to monitor BCAs; in particular, those based on conventional PCR give extremely sensitive and specific detection (El Hamouchi *et al.* 2008; Pujol *et al.* 2006). In the present work, a molecular identification of CPA-2 based on a pair of SCAR primers developed and applied in citrus fruit in a previous work was used to monitor *P. agglomerans* CPA-2 during postharvest application on apples (Nunes *et al.* 2008). During the monitoring of CPA-2 postharvest application on apples, the results showed that the persistence of CPA-2 was only 1 day after treatment. CPA-2 was not detectable in the treatment area environment. This low dispersion of CPA-2 in the environment may be due to the fact that the drencher used was automatically closed during the treatment, providing a closed system. CPA-2 was not detectable in the environment or on different storage chamber surfaces at either 20 °C or 0 °C. In contrast, on plastic boxes, CPA-2 persisted no more than 5 days at 0 °C. The persistence of CPA-2 on working clothes was not longer than 3 days.

Our results demonstrate that *P. agglomerans* CPA-2 has a low impact because it did not grow and disperse in the environment, which is very important for registration issues (Alabouvette and Cordier, 2011). Due to its low establishment, survival and dispersal in the environment, CPA-2 does not present an unacceptable risk for the environment. The persistence of CPA-2 on the different surfaces sampled could be limited by nutrient restriction and the accumulation of metabolic products in the medium that causes growth inhibition (Ohara *et al.* 1992).

Molecular methods based on PCR are extremely sensitive and specific for nucleic acid detection, but conventional PCR does not provide effective quantification. Recently, methods based on qPCR have become more widespread for to be extremely sensitive, highly specific and most importantly, quantitative detection of BCAs in general and particularly (Braun-Kiewnick *et al.* 2012; Larena and Melgarejo, 2009; Massart *et al.* 2005; Spotts *et al.* 2009). Moreover, qPCR offers several advantages over the traditional dilution plating method for the quantification of *P. agglomerans* strain CPA-2. qPCR possesses the potential to detect a single target molecule in a complex mixture, is rapid, versatile and, depending on the design of primers, enables strain-level detection which can be utilized to sensitively and accurately detect specific BCAs and monitor their population dynamic over a period of time (Sanzani *et al.* 2014; Schena *et al.* 2004).

The primer pair SP_2-F/SP_2-R and a *Taqman* MGB probe developed from a variable region of *P. agglomerans* CPA-2 DNA. This set proved to be specific when tested by qPCR of purified DNA from CPA-2, 17 strains of *P. agglomerans*, 4 related *Pantoea* species, and 21 bacterial strains from other genera isolated from fruit and vegetables yielded amplification of only CPA-2. Furthermore, the system enabled the amplification approximately 10^3 cells per reaction of CPA-2 (average *Cq* value of 35.04). Outside of these values, linearity was lost, or no signal was detected. A slightly lower detection limit of 10^4 CFU/50 mL with an average *Cq* value of 38.16 was reported by Massart *et al.* (2005) for the specific detection and monitoring of *Candida oleophila* (strain O) using a *Taqman* MGB probe corresponding to the same detection method used in our study. *Taqman* MGB probes allow the use of shorter, highly specific oligonucleotide sequences (Afonina *et al.* 1997), which should be suitable for amplification of highly fragmented DNA and especially when a mismatch occurs in the MGB region of duplex (Kutyavin *et al.* 2000).

The quantification of *P. agglomerans* CPA-2 on apples under two different storage conditions at 20 °C and 0 °C was determined by two methods, qPCR and dilution plating. In general, the results showed that: *(i)* during the short storage time, both methods showed changes in population levels, but the data were not differences, and *(ii)* during the long storage time, qPCR showed an overestimation of the population of CPA-2. Spotts *et al.* (2009) also observed a higher correlation between concentrations of DNA, $\mu g/m^2$ determined with qPCR and CFU/L of *Cytofilobasidium infirmominiatum* on fruit recently treated with this BCA. Studies conducted by Pujol *et al.* (2006) confirmed the differences in population level estimated by qPCR and dilution plating; the estimation of the population levels of *Pseudomonas fluorescens* EPS62e NaL by qPCR was approximately 1,700 times higher than those CFU counts obtained by dilution plating from leaves of apple trees. These authors supposed that these differences were due to the stressful conditions of the leaf environment that promote entry into a viable but

nonculturable cell state (VBNC), the presence of cell aggregates that were not dispersed before plating, or the presence of free DNA after cell death. In our study, the presence of CPA-2 cell aggregates in the washing suspension was unlikely because of the high efficacy of the homogenization method used (Torres *et al.* 2012). The differences in quantification between the two methods at initial times of storage are likely due to the induction of a VBNC state, or at long storage times, may be due to the presence of nondegraded DNA in our samples. Therefore, further studies should be done in this sense. Research has evaluated DNA persistence in soil, but in some studies, DNA was rapidly degraded (Schena and Ippolito, 2003), whereas in others, the DNA persisted for a long period of time after cell death, resulting in an overestimatation of the total number of microorganisms due to the point detection of dead and live cells (England *et al.* 1997). To overcome this problem, the coupling of qPCR with the use of nucleic acid intercalating dyes such as propidium monoazide (PMA) could be possible.

1.5. CONCLUSIONS

This study describes the development of a qPCR method to identify and quantify *P. agglomerans* CPA-2 applied on postharvest of apples. This method is novel and is a potential tool to quickly and specifically monitor populations of CPA-2 in large-scale experiments immediately after their application. The possibility to use more efficient DNA-based methods such as qPCR greatly facilitates BCA fate and activity after application. The persistence of *P. agglomerans* CPA-2 on surface areas and the environment after postharvest application were low, which is very important for its future application in packinghouses. In general, these advances provide new possibilities for insights into ecophysiology constraints and can be used to generate valuable data for registration purposes.

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CAPÍTULO II

Development of PMA real-time PCR method to quantify viable cells of *Pantoea agglomerans* CPA-2, an antagonist to control the major postharvest diseases on oranges

Soto-Muñoz, L., Teixidó, N., Usall, J., Viñas, I., Crespo-Sempere, A., Torres, R.

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ABSTRACT

Dilution plating is the quantification method commonly used to estimate the population level of postharvest biocontrol agents, but this method does not permit a distinction among introduced and indigenous strains. Recently, molecular techniques based on DNA amplification such as quantitative real-time PCR (qPCR) have been successfully applied for their high strain-specific detection level. However, the ability of qPCR to distinguish viable and nonviable cells is limited. A promising strategy to avoid this issue relies on the use of nucleic acid intercalating dyes, such as propidium monoazide (PMA), as a sample pretreatment prior to the qPCR. The objective of this study was to optimize a protocol based on PMA pre-treatment samples combined with qPCR to distinguish and quantify viable cells of the biocontrol agent *P. agglomerans* CPA-2 applied as a postharvest treatment on orange. The efficiency of PMA-qPCR method under the established conditions (30 μM PMA for 20 min of incubation followed by 30 min of LED light exposure) was evaluated on an orange matrix. Results showed no difference in CFU or cells counts of viable cells between PMA-qPCR and dilution plating. Samples of orange matrix inoculated with a mixture of viable/dead cells showed 5.59 $\log_{10}$ CFU/mL by dilution plating, 8.25 $\log_{10}$ cells/mL by qPCR, and 5.93 $\log_{10}$ cells/mL by PMA-qPCR. Furthermore, samples inoculated with heat-killed cells were not detected by dilution plating and PMA-qPCR, while by qPCR was of 8.16 $\log_{10}$ cells/mL. The difference in quantification cycles *(Cq)* among qPCR and PMA-qPCR was approximately 16 cycles, which means a reduction of 65536 fold of the dead cells detected. In conclusion, PMA-qPCR method is a suitable tool for quantify viable CPA-2 cells, which could be useful to estimate the ability of this antagonist to colonize the orange surface.

Keywords: biocontrol, fruit, quantitative method, qPCR, PMA, viable cells.

2.1. INTRODUCTION

Biological control using microbial antagonists has emerged as one of the most promising alternatives to postharvest applications of chemical fungicides (Janisiewicz and Korsten, 2002). Specifically, the antagonist activity of the bacterium *Pantoea agglomerans* strain CPA-2 against blue and green decays in citrus fruit under pre- and postharvest conditions has been clearly demonstrated (Cañamás *et al.* 2008a; Plaza *et al.* 2004; Teixidó *et al.* 2001; Torres *et al.* 2007; Usall *et al.* 2008) and the formulated product ready to apply commercially, is now available (Torres *et al.* in press).

The main goal of the development and implementation of a biocontrol product is to improve the ability of the biological agent (BCA) to successfully control postharvest diseases under a wider array conditions and with minimal variability (Droby *et al.* 2003). An effective decay control depends on the ability of antagonist to colonize the surface of fruit both in the field and in storage and to persist for as long as possible is vitally important (Wisniewski and Wilson, 1992). The success of biocontrol activity may also be influenced by the tolerance of BCA to the environmental stresses such as dry conditions, direct UV irradiation, high temperatures, low nutrient availability, and rapid climatic changes (Nunes, 2012). It is known that it is possible to adapt a microorganism to unfavorable environmental by induction of stress responses. Studies conducted by Cañamás *et al.* (2008a,b) on the antagonist *P. agglomerans* CPA-2 observed that treatments with osmotic-adapted and lyophilized cells exhibited greater survival rates than with non-osmotic-adapted or fresh cell under field condition.

The antagonist applications require specific monitoring strategies for the careful evaluation of the antagonist's ability to colonize fruits either externally or internally, tolerate climate changes, or be active against target pathogens (Gullino *et al.* 1995). A wide range of methods have been developed to monitor microbial populations. Dilution plating is the most frequently used method for monitoring and counting microbial antagonist (Schena *et al.* 2002). This method is valuable but labor-intensive and does not permit any distinction between introduced and indigenous strains (Llop *et al.* 1999;

Schena *et al.* 2004). Moreover, it may underestimate the actual population size because bacteria could enter in a viable but nonculturable (VBNC) state. The VBNC state represents a transient inability to grow on nutrient medium, on which bacteria normally grow and develop colonies, while still being metabolically active (Oliver, 2010).

Molecular methods targeting nucleic acid have revolutionized BCAs detection (Sanzani *et al.* 2014). Recently more specific, sensitive and rapid monitoring tools have been developed for BCAs based on DNA amplification such as quantitative real-time PCR (qPCR), which have been increasingly reported for several BCAs (Braun-Kiewnick *et al.* 2012; Larena and Melgarejo, 2009; Massart *et al.* 2005; Pujol *et al.* 2006; Spotts *et al.* 2009). Despite these advantages, the broad application is still hampered by some challenges. One limitation of qPCR lies in its inability to differentiate between viable and non-viable cells and the resulting overestimation of microbial targets (Fittipaldi *et al.* 2012).

In the environment extracellular DNA can persist in different matrices for varying durations depending on matrix properties (Levy-Booth *et al.* 2007). Previous work conducted in our laboratory showed that apples treated with *P. agglomerans* CPA-2 and stored at 20 °C or 0 °C, the quantification by qPCR had the advantage to detect cultivable and VBNC cells, for short storage times, in relation to dilution plating. However, for a long storage times, qPCR had limitation of overestimation of the population of CPA-2, may be due to the presence of nondegraded DNA on fruit surface after cell death (Soto-Muñoz *et al.* 2014).

A promising strategy to avoid this issue relies on the use of nucleic acid intercalating dyes, such as propidium monoazide (PMA), as a sample pretreatment prior to qPCR (Nocker *et al.* 2006). PMA has been reported to only penetrate cells with compromised membranes and once inside the cell, PMA intercalates into the DNA to which it can be covalently cross-linked upon exposure to light. This irreversible DNA modification results in suppression of PCR amplification (Nocker *et al.* 2006; Nocker *et al.* 2007; Nogva *et al.* 2003). Although the dye is membrane-impermeable in viable cells, environmental stresses increase the permeability of the cell wall in killed bacteria to PMA (Pribylova *et al.* 2012). Consequently, PMA treatment combined with qPCR (PMA-qPCR) has been successfully tested in simple matrices such as pure and mixed cultures on food microorganisms such as *Listeria monocytogenes* (Pan and Breidt, 2007), *Escherichia coli* O157:H7 (Elizaquível *et al.* 2012; Nocker and Camper, 2009) and *Campylobacter jejuni* (Josefsen *et al.* 2010). Not only PMA has been applied to discriminate between dead and live bacteria; it has been also used to identify viable yeasts (Andorra *et al.* 2010) and fungi (Crespo-Sempere *et al.* 2013). However, the application of the PMA approach for detection of BCAs applied on fruit to control postharvest diseases has not

been evaluated yet. In addition, for each matrix an optimization the several variables such as: concentration of PMA, light exposure time, and dilution of suspended solids to a concentration conducive to photoactivation of PMA is needed (van Frankenhuyzen *et al.* 2011). In this report, we have developed a specific PMA treatment combined with qPCR to quantify viable cells of *P. agglomerans* CPA-2 on oranges as a postharvest treatment to control their major diseases.

2.2. MATERIAL AND METHODS

2.2.1. Culture conditions of CPA-2

The CPA-2 strain of *P. agglomerans* used in this study was obtained from IRTA in Lleida (Catalonia, Spain). This strain was isolated from *Golden Delicious* apples and is currently deposited at the Spanish Collection of Type Culture (CECT, University of Valencia, Valencia, Spain), as CECT-4920. Stock cultures were stored long-term at -80 °C in glass beads and subcultured on nutrient yeast dextrose agar (NYDA: 8 g/L nutrient broth, 5 g/L yeast extract, 10 g/L dextrose and 20 g/L agar). The activated culture was maintained on NYDA at 30 °C for 24 h and transferred to a potassium phosphate buffer (pH 6.5) (0.2 M KH_2PO_4, 70 mL; 0.2 M K_2HPO_4, 30 mL and deionised water, 300 mL) to obtain a cell suspension. This suspension was used as an inoculum for biomass production in a fermentation system. Osmotically adapted cells grown in basal liquid medium (5 g/L yeast extract, 10 g/L sucrose and 25 g/L NaCl), as previously described by Cañamás *et al.* (2008b), were used in all studies. An appropriate volume of inoculum was added to 5 L of the basal liquid medium adjusted to 1 x 10^6 CFU/mL. Cultures were grown in a 5 L bench-top fermenter BIOSTAT-A (Braun Biotech International, Mesungen, Germany) at 30 °C with 300 rpm agitation and 100 L/h aeration. Cultures were harvested at stationary phase (24 h) by centrifugation at 9820 *g* for 10 min at 15 °C in an Avanti™ J-25 centrifuge (Beckman, Palo Alto, Ca, USA). Cell pellets were resuspended in deionised water containing 10 % sucrose, frozen at -20 °C overnight and freeze-dried (Cryodos, Telstar SA, Terrasa, Catalonia, Spain) at 1 Pa and -45 °C for 24 h as described by Costa *et al.* (2000). These lyophilised cells were packaged in a white high-density polyethylene bottle at non-vacuum atmosphere (Torres *et al.* unpublished results). Cell pellets were resuspended in 1 % non-fat skim milk (Sveltesse, Nestle, Vevey, Switzerland) and used immediately for all assays.

2.2.2. Cell suspensions and killing conditions

The cell suspensions consisted of: *(i)* 100 % viable cells, *(ii)* 100 % dead cells; and (*iii*) a mixture of 75 % of viable cells with 25 % of dead cells. Cell suspensions were adjusted

to approximately 10^8 CFU/mL in potassium phosphate buffer. The dead cells were heat-killed, autoclaved for 15 min at 121 °C and 15 psi and then cooled at room temperature. The cell viability was conducted by plating on NYDA media and incubation for 24 h at 30 °C. Loss of viability of dead cells was confirmed with a pre-enrichment on nutrient yeast dextrose broth (NYDB: 8 g/L nutrient broth, 5 g/L yeast extract and 10 g/L dextrose) at the optimal growth conditions followed by plating on NYDA media and incubating for 24 h at 30 °C.

2.2.3. Effect of PMA concentration

PMA (phenanthridium, 3-amino-8-azido-5-[3- (diethylmethylammonio)propyl] -6-phenyl dichloride; Biotum, Hayward, USA) was dissolved in 20 % dimethyl sulfoxide (DMSO) (Sigma-Adrich, St. Louis, MO) to obtain a stock solution of 20 mM and stored at -20 °C (Nocker *et al.* 2007). PMA stock solution was added to 500 µL of each cell suspension (equilibrate to room temperature) to obtain a final PMA concentration of 30 or 50 µM.

After the addition of the reagent, an incubation period of 20 min in the dark at room temperature was performed with constant agitation at 250 rpm (VMR mini shaker). Thereafter, PMA-treated cell suspensions were exposed to light using a photo-activation system during 20 min. A closed box with refectory walls wells and blue wavelength light-emitting diodes (LED 5 mm, 3.7 v, 20 mA, 2600 mcd) was constructed and placed 7 cm from a 24-well microplate containing the cell suspensions. Afterward photo-induced cross-linking, cells were centrifuged at 9727 *g* for 20 min and supernatant was removed. The resulting pellets were used for DNA extraction, followed by qPCR as described in Section 2.2.7. Non-PMA treated cell suspensions were used as a negative control. Each experimental condition was repeated in triplicate to ensure reproducibility of results.

As for all subsequent experiments, the effect of PMA was expressed as 'signal reduction' obtained by subtraction of quantification cycles values *(Cq)* of non-PMA treated cell suspensions from the *Cq* values of the corresponding PMA-treated cell suspensions (negative control). To evaluate the toxicity of PMA on *P. agglomerans* cells, PMA-treated viable cells were enumerated by dilution plating on NYDA media.

2.2.4. Effect of PMA incubation time

PMA stock solution was added to 500 µL of each cell suspension (equilibrate to room temperature) to obtain a final PMA concentration of 30 µM. PMA-treated cells were homogeneously mixed and incubated in the dark at room temperature for 5, 20, or 40 min with constant agitation at 250 rpm. Subsequently, PMA-treated cells were

exposed to LED light during 20 min. Afterward photo-induced cross-linking, cells were centrifuged at 9727 *g* for 20 min prior to DNA extraction. The resulting pellets were used for DNA extraction, followed by qPCR as described in Section 2.2.7. Non-PMA treated cell suspensions were used as a negative control. Each experimental condition was repeated in triplicate to ensure reproducibility of results and the effect of PMA was expressed as 'signal reduction'.

2.2.5. Effect of LED light exposure time

In order to assess the effect of LED light exposure time on each suspension of *P. agglomerans* cells, PMA stock solution was added to 500 µL aliquots of cell suspension (equilibrate to room temperature) to obtain a final PMA concentration of 30 µM. PMA-treated cells were homogeneously mixed and incubated in the dark at room temperature for 20 min with constant agitation at 250 rpm. Subsequently, PMA-treated cells were exposed to LED light source during 10, 20 or 30 min. Afterward photo-induced cross-linking, cells were centrifuged at 9727 *g* for 20 min prior to DNA extraction. The resulting pellets were used for DNA extraction, followed by qPCR as described in Section 2.2.7. Non-PMA treated cell suspensions were used as a negative control. Each experimental condition was repeated in triplicate to ensure reproducibility of results and the effect of PMA was expressed as 'signal reduction'.

2.2.6. Effect of the orange matrix on the PMA-qPCR method

Valencia Oranges (*Citrus sinensis* cv *Valencia late*) were harvested at commercial maturity from Baix Ebre-Montsià areas in Tarragona (Catalonia), selected by hand for uniformity of size and ripeness. Fruits were stored without any chemical postharvest treatment at 4 °C and 85 % relative humidity (RH) before use.

Orange peel samples, considered as orange matrix, were obtained according to the method described by Torres *et al.* (2012) to recover *P. agglomerans* CPA-2 from orange surface with a minor modifications. Briefly, 40 pieces of peel were removed from five fruits using a cork borer with a diameter of 16 mm. Peel samples were dipped into 20 mL of phosphate buffer solution. Then, samples were inoculated with different *P. agglomerans* suspensions of: *(i)* viable cells, *(ii)* dead cells, and *(iii)* a mixture of 75 % of viable cells with 25 % of dead cells as described in Section 2.2.2. All inoculations were carried out in triplicate. A non-inoculate sample was included in each experiment (negative control). Each orange matrix, previously inoculated with CPA-2, was homogenized in a Stomacher 400 (Seward, London, UK) set at normal speed for 90 s. Eight milliliters of the resulting suspension were filtered through a 20 mm porous filter (Whatman® International, Maidstone England) and split in two equivalent samples. One of them was untreated and the other one was treated with 30 µM PMA, 20 min of

incubation and 30 min of LED light exposure. All samples were centrifuged at 9727 *g* for 20 min and the resulting pellets were used for DNA extraction, followed by qPCR as described in Section 2.2.7.

Moreover, each orange matrix inoculated with *P. agglomerans* cells was quantified by dilution plating on NYDA media. The colonies were counted after incubation at 30 °C for 24 h. All experiments were repeated twice in triplicate to ensure reproducibility of results.

2.2.7. DNA extraction and qPCR

Genomic DNA of *P. agglomerans* CPA-2 was extracted using Lyse-N-Go PCR Reagent (Thermo Scientific, Rockford, IL, USA) following the manufacturer's instructions. The amount and purity of DNA samples were determined spectrophotometrically (NanoDrop ND-1000; NanoDrop Technologies, Wilmington, DE) and DNA integrity was analysed by electrophoresis on 1.5 % agarose gel run at 70 V for 90 min with TEB buffer and stained with 0.5 mg/µL ethidium bromide (Sambrook and Russell, 2001).

The ABI-7500 qPCR detection system (Applied Biosystem Division, Perkin-Elmer Co. Foster City, CA, USA) was used to quantify genomic CPA-2 DNA. Each qPCR was performed in MicroAmp optical 96-well plates that were sealed with an optical adhesive cover (Applied Biosystem, Foster City, CA, USA). PCR reactions were performed in a final volume of 25 µL containing 1× PCR *Taqman* Universal PCR Master Mix (Applied Biosystem, USA), 300 nM of SP_2-forward primer (5'-CTTAAAGCGCAGGGAAGCCGGTCAG-3'), 300 nM of SP_2-reverse primer (5'-GAGCCGGCTCAGGGAAACCGGTC-3'), 200 nM of *Taqman* MGB probe (FAM-TCCATGGATGGCTTAAG-MGB) defined a previous work (Soto-Muñoz *et al.* 2014) and 4 µL of DNA extracted with and without use of PMA treatment diluted 10-fold. Cycling conditions used in this study were: 2 min at 50 °C, 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 59 °C. Reactions were done in triplicate. In all cases, a negative amplification control was included using 4 µL of water instead of genomic DNA. Data acquisition and analysis were performed using SDS software version 1.3.1 (Applied Biosystems) according to the manufacturer's instructions. The *Cq* value were automatically calculated for each reaction by the SDS software. Any sample that reached a fluorescence value exceeding the fluorescence threshold value was considered positive. For the determination of the sensitivity and the amplification efficiency of the qPCR reactions, a standard curve was constructed for *P. agglomerans*. A standard quantification curve was prepared by adding a range of bacterial cell concentrations (from 2×10^8 to 2×10^4 cells/mL) to orange matrix. Genomic DNA was extracted and amplified as described above. The *Cq* value that determines the cycle number at witch fluorescence increases above background, was plotted against the logarithm of starting

quantity of template for each dilution. Amplification efficiency was calculated from slope of the standard curve E= $10^{-1/slope}$; % Efficiency = (E-1) x 100.

2.2.8. Statistical analysis

The significance of difference between the signal reduction and the suspensions of viable, dead and mixture of viable/dead cells of *P. agglomerans* were analyzed using analysis of variance (ANOVA) with JMP®8 statistical software (SAS Institute, Cary, NC, USA). Statistical significance was judged at the level $P<0.05$. When the analysis was statistically significant, the Student's *t*-test for separation of means was used.

2.3. RESULTS

2.3.1. Effect of PMA concentration

The different cell suspensions (viable, dead and a mixture of viable/dead cells) were exposed at 30 or 50 µM of PMA concentrations. The results showed that both concentrations did not show significant difference on the signal reduction of qPCR amplification in viable cells and the mixture of viable/dead cells. A slight signal reduction was observed in dead cells treated with 50 µM compared to those treated with 30 µM, but these differences were not statistically significant ($P>0.05$) (Figure 1).

Furthermore, no toxic effect was observed when viable cells were treated at 30 µM PMA. However, difference in CFU counts was found in 50 µM PMA-treated cells (around 0.40 log). According to this result, the PMA concentration of 30 µM was chosen to guarantee an efficient inactivation of DNA from dead cells and avoid a toxic effect on viable cells.

2.3.2. Effect of PMA incubation time

The signal reduction of PMA-treated cells compared to the DNA amplification of non-PMA treated cells depending on incubation time is shown in figure 2. Results showed that PMA-treated viable cells and incubated for 5, 20, or 40 min did not show significant difference effect on the signal reduction of qPCR amplification. The maximal signal reduction was approximately 2.0 cycles after 40 min incubation time with PMA dye.

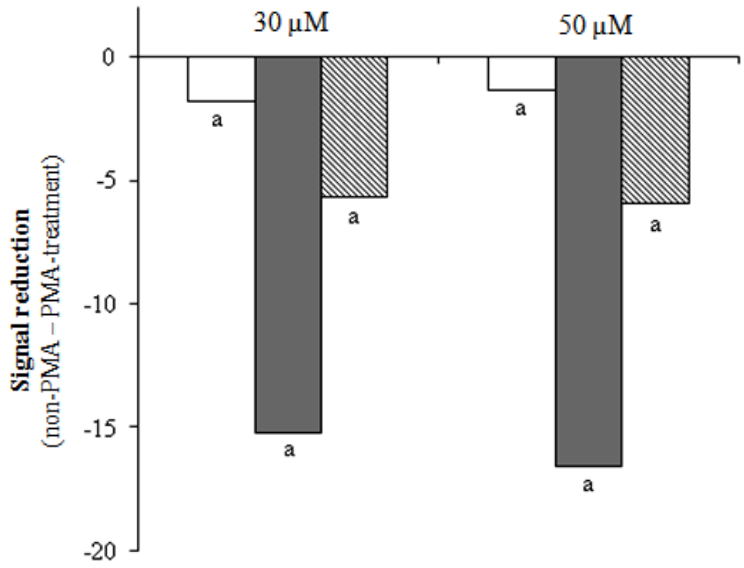

Figure 1. Effect of PMA concentration on PMA-qPCR signals of: viable (□), dead (■), and mixture of viable/dead (▧) cells of *P. agglomerans* CPA-2. Signal reductions were derived by subtracting *Cq* values obtained from non-PMA treated cell aliquots from the *Cq* values from PMA-treated cells (negative control). *P. agglomerans* cells were exposed to 30 or 50 µM PMA during 20 min of incubation and 20 min of exposure LEDs light. Means with the same letter for each cell suspension are not significantly different ($P<0.05$) according to Student's *t*-test. Each column represents the mean of three independent replicates.

PMA-treated dead cells showed a signal reduction around 3.0 cycles after 5 min of incubation time. A notable signal reduction of 16.5 cycles was observed at 20 min of incubation. However, after 40 min was not observed increment of the signal reduction statistically significant when compared with 20 min of incubation time.

Moreover, an incubation period of 5 min allowed a signal reduction of 0.84 cycles for mixture of viable/dead cells. Although, the maximal signal reductions were of 5.30 and 5.60 cycles at 20 and 40 min, respectively.

2.3.3. Effect of LED light exposure time

Exposure periods for 10, 20 or 30 min to LED light was tested in suspensions of viable, dead and a mixture of viable/dead cells of *P. agglomerans* CPA-2 previously treated with 30 µM PMA and incubated for 20 min (Figure 3). PMA-treated viable cells showed a signal reduction of 1.53, 1.91, and 1.38 cycles after of 10, 20 and 30 min of LED

light exposure, respectively. These signal reductions did not were statistically different comparing between the exposures times evaluated.

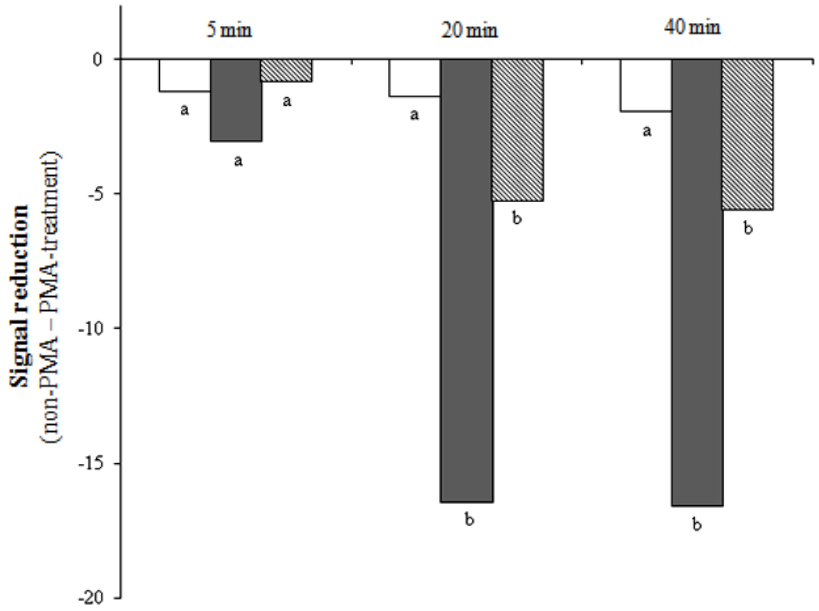

Figure 2. Effect of PMA incubation time on PMA-qPCR signals of: viable (□), dead (■), and mixture of viable/dead (▨) *P. agglomerans* CPA-2 cells. Signal reductions were derived by subtracting *Cq* values obtained from non-PMA treated cell aliquots from the *Cq* values from PMA-treated cells (negative control). *P. agglomerans* cells were exposed to 30 µM PMA during 5, 20, or 40 min of incubation and 20 min of exposure LEDs light. Means with the same letter for each cell suspension are not significantly different ($P<0.05$) according to Student's *t*-test. Each column represents the mean of three independent replicates**.**

PMA-treated dead cells, the signal reduction resulted significantly different among the incubation times. A negligible signal reduction of 0.36 cycles was observed after 10 min to exposure to LED light, suggesting that PMA did not bind to DNA of dead cells. In contrast, an increased in LED light exposure times from 20 to 30 min, incremented significantly the signal reduction from 15.80 to 18.00 cycles, respectively. However, 10 min of LED light exposure time showed a lower signal reduction of 0.40 cycles in PMA-treated mixture of viable/dead cells. In addition, the signal reduction obtained after 20 or 30 min of LED light exposure was stronger than after 10 min exposure, with a difference of 4.30 and 8.30 cycles, respectively. Taking into account these data, 30 min of LED light exposure was chosen to guarantee efficient cross-linking of PMA to DNA.

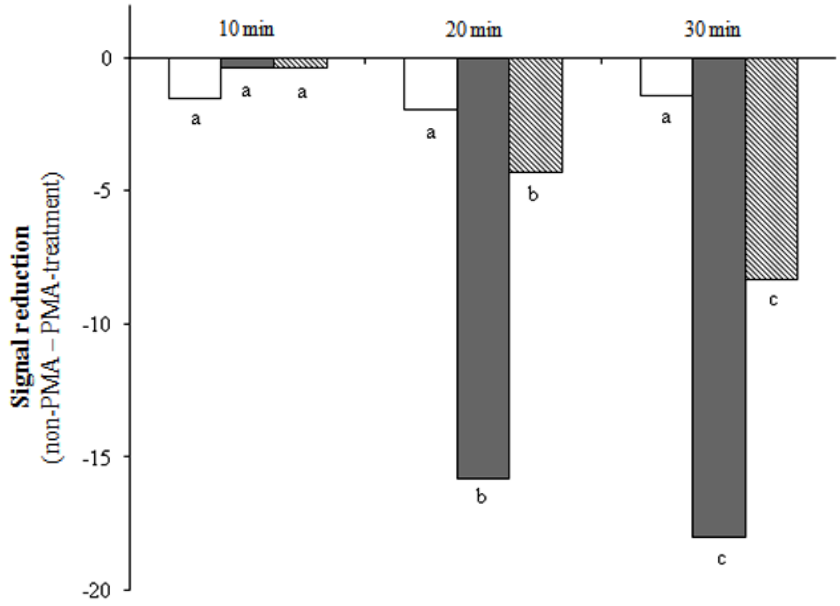

Figure 3. Effect of LEDs light exposure on PMA-qPCR signals of: viable (□), dead (■), and mixture of viable/dead (▨) *P. agglomerans* CPA-2 cells. Signal reductions were derived by subtracting *Cq* values obtained from non-PMA treated cell aliquots from the *Cq* values from PMA-treated cells (negative control). *P. agglomerans* cells were exposed to 30 µM PMA during 20 min of incubation and 10, 20, or 30 min of LED light exposure. Error bars represent standard deviations from three replicates. Means with the same letter for each cell suspension are not significantly different ($P<0.05$) according to Student's *t*-test. Each column represents the mean of three independent replicates**.**

2.3.4. Effect of the orange matrix on the PMA-qPCR

The application of PMA-qPCR method under the conditions previously established (30 µM PMA, 20 min of incubation and 30 min of LED light exposure) was evaluated on an orange matrix. The standard curve showed a strong linear relationship (0.996) among the DNA extracted from the orange matrix inoculated with different CPA-2 concentrations (10^8, 10^7, 10^6, 10^5 and 10^4 cells/mL) and *Cq* values (Figure 4). When *Cq* values were plotted against cell concentrations, a slope of -3.088 was obtained, indicating an efficiency of 2.09 (109 %).

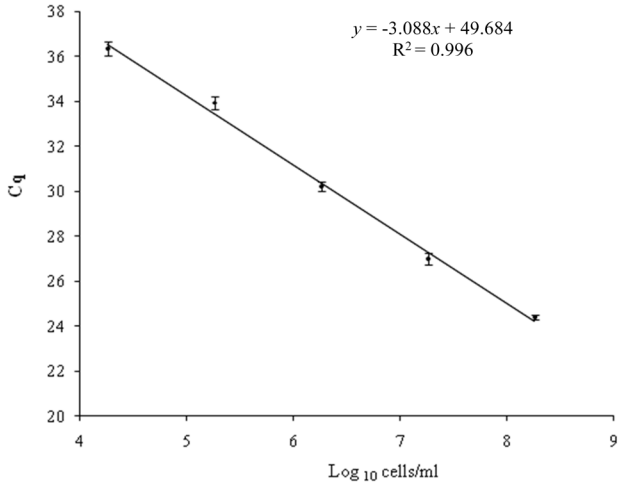

Figure 4. Real-time PCR amplification plots for reactions with genomic DNA templates of mixed bacteria (plots 1-5: ranged from 10^8 to 10^4 cells/qPCR). DNA templates were extracted from serial diluted mixed bacterial cells of an orange matrix

Suspensions of *P. agglomerans* viable, dead and mixture of viable/dead cells were resuspended in the orange matrix and then quantified by various methods: *(i)* dilution plating to obtain the active-culturable population, *(ii)* qPCR to obtain the total cell count, and *(iii)* PMA-qPCR treatment to obtain viable cell counts (Table 1). Results showed bacterial counts of 8.08, 8.23 and 8.02 $\log_{10}$ CFU or cells/mL for dilution plating, qPCR and PMA-qPCR, respectively in orange matrix inoculated with viable cells. Counts for orange matrix inoculated with dead cells were not detected using dilution plating and PMA-qPCR, while by qPCR was of 8.16 $\log_{10}$ cells/mL. Counts of orange matrix inoculated with a mixture of viable/dead cells were 5.59 $\log_{10}$ cells/mL, 8.25 $\log_{10}$ CFU/mL and 5.93 $\log_{10}$ CFU or cells/mL for dilution plating, qPCR and PMA-qPCR, respectively.

Table 1. Quantification of viable, dead and viable/dead mix of *P. agglomerans* CPA-2 cells resuspended in orange matrix by dilution plating, qPCR and PMA-qPCR methods.

Sample CFU/mL		Plate count	qPCR	PMA-qPCR
Viable cells	Dead cells	$\log_{10}$ CFU/mL	$\log_{10}$ cells/mL (*Cq*)	$\log_{10}$ cells/mL (*Cq*)
10^8	-	8.08 ± 0.10	8.23 ± 0.03(24.03±0.09)	8.02 ± 0.04 (24.68±0.14)
-	10^8	not detected	8.16 ± 0.04 (24.23±0.14)	not detected (> 40)
10^6	10^2	5.59 ± 0.03	8.25 ± 0.04 (23.94±0.13)	5.93 ± 0.02 (31.07±0.06)

Samples are an average of three independent replicates ± standard deviation.

In contrast, *Cq* values obtained in orange matrix inoculated with viable cells were 24.03 and 24.68 cycles, by qPCR and PMA-qPCR, respectively. Samples of orange matrix inoculated with dead cells showed a difference of approximately 16.0 cycles among *Cq* values obtained by qPCR and those obtained by PMA-qPCR. This difference indicates an important reduction of dead cell detection around 65536 fold. Finally, orange matrix inoculated with a mixture of viable/dead cells showed a *Cq* value of 23.94 and 31.07 cycles by qPCR and PMA-qPCR, respectively.

2.4. DISCUSSION

Effectiveness of a BCA is closely related to the concentration of cells in the treatment suspension and its survival in the surface of the fruit (Chand-Goyal and Spotts, 1996). Population dynamic studies involve traditionally dilution plating (De Cal *et al.* 2009; Janisiewicz and Jeffers, 1997; Teixidó *et al.* 1999). However, this method is subject to several limitations including contamination by other microorganisms common in orchard and packinghouse environments, and an incubation period of at least 48 h before colonies can be counted (Spotts *et al.* 2009). In the present study, we have evaluated the applicability of PMA-based viable-dead distinction method for the selective detection and quantification of antagonist *P. agglomerans* CPA-2 on orange surface.

Initially, viable, dead and a mixture of viable/dead cell suspensions were exposed to two different PMA concentrations. The reduction signal was not significantly different between both tested PMA concentrations, 30 and 50 μM. However, the treatment with 50 μM PMA showed a toxic effect on *P. agglomerans* cells. A minor cytotoxic effect was also observed by Pan and Breidt (2007) when preconditioned mixtures of different strains of *L. monocytogenes* for 2 h at different no-lethal temperatures (5, 15, 25, and 37 °C) were treated with 50 μM of PMA. In addition, these authors reported that the minimal inhibitory concentration (MIC) of PMA was 1600 μM. A similar experiment was performed with *Legionella pneumophila* by Yañez *et al.* (2011): aliquots of live *L. pneumophila* suspensions were pre-conditioned to different non-lethal temperatures (4, 22, 35, and 44 °C) for 2 h followed by treatment with 100 or 200 μM PMA and 5 min of light exposure. Results indicated that 100 μM PMA treatment only minimally reduced colony counts (compared to the non-treated controls). Slightly higher cytotoxicity was observed at 200 μM and showed the strongest reduction in colony numbers with cells preconditioned at 44 °C around 0.44 log units.

The effect of PMA incubation time was evaluated on viable, dead and a mixture of viable/dead cells of *P. agglomerans*. Our results showed a substantial increase in signal reduction at 20 min of incubation. Furthermore, prolonged incubation times (40 min) did not increase the signal reduction in dead cells, and only showed no significant effect

on viable cells. Similar findings have been reported for *Mycobacterium avium* subsp. *paratuberculosis* (Kralik *et al.* 2010); in this study the effect of different incubation times (5, 20, and 50 min) on PMA-qPCR signals of live and heat-killed cells revealed that incubation times longer than 20 min not only increased *Cq* values of dead cells, but also *Cq* values of live cells. The authors concluded that the observed signal reductions with untreated cultures could be due to either the presence of membrane-compromised cells or the possibility that PMA might also enter live cells to a certain extent, or a combination of both. For bacterial studies, an incubation of 5 min is commonly used (Chen *et al.* 2011; Nocker *et al.* 2006; Varma *et al.* 2009). However, incubation times have to be seen in context with the target microbial species and the applied dye concentration. An alternative to a prolonged dye incubation time in the case of microorganism with a low dye penetration rates might be useful. Using low dye concentrations, incubation time can be more flexible (Fittipaldi *et al.* 2012).

The effect of LED light exposure time was evaluated on viable, dead and a mixture of viable/dead cells. A period of 30 min showed a major increase in *Cq* values of dead cells, which means a lower detection of dead cells. A light exposure of dye treated samples is important for: *(i)* activation of acid nucleic-bound dye and *(ii)* inactivation of excess dye that has not entered cells and that could potentially bind to DNA from lives cells during the DNA extraction procedure (Nocker *et al.* 2006; Nocker *et al.* 2007). Due to the great diversity of light sources used in previous works, it is quite difficult to compare our results with those obtained by other authors, due to the wide variety of lamps and differences in exposure procedures. Initial researches with viability dyes employed photoactivation of viability dyes using high-power lamps (500-750 W halogen lamps). However, a disadvantage of the halogen lamps is an intense emission of heat and experimental variation. An alternative to halogen lamps is the use of LEDs (Vesper *et al.* 2008). In comparison with halogen lamps, LEDs have a decisive advantage in emitting light of a defined specific wavelength allowing for optimal dye activation and avoiding the generation of heat (Fittipaldi *et al.* 2012).

The last variable to consider is the matrix where cells will be suspended; it has important consequences for the uptake of PMA (Nkuipou-Kenfack *et al.* 2013). In this work, the efficiency of PMA-qPCR method was evaluated on an orange matrix. In general, no differences in bacterial counts of viable cells were observed between PMA-qPCR and dilution plating. Martinon *et al.* (2012) observed the same trend in results obtained from dilution plating, qPCR and PMA-qPCR for quantifying *E. coli*, *Staphylococcus aureus* and *L. monocytogenes* in food-grade steel surface. For these three bacterial species after storage times of 30 or 60 min on contaminated surfaces, authors found differences between qPCR and dilution plating, as well as between qPCR and PMA-qPCR. Authors suggested that the discrepancies among these three methods

might in part be explained by the presence of a heterogeneous population of live, dead, non-viable or VBCN cells within a swab sample. In our study, the observed signal reductions with viable cells samples could either be due presence of a certain proportion of membrane-compromised cell, especially considering that of *P. agglomerans* cells has been subjected to a freeze-drying process. This process combines a number of stresses that may cause injury to microorganisms and can alter permeability cytoplasm membrane and also damage cell wall (Sinskey and Silverma, 1970). Thereby, it is reasonable to speculate that PMA may also bind DNA from injured cells. These damaged cells may repair their membranes and may be present as VBCN cells in samples as viable cells (Knight, 2000). In contrast, a reduction of 65536 fold of dead cell detected from samples inoculated with dead cells and treated with PMA. This reduction was higher than reported by Schmidlin *et al.* (2010), where a insufficient distinction between DNA from viable and non viable *S. aureus* cells in wape-samples were observed by use of PMA-qPCR, it did reduce amplification of DNA extracted from dead bacteria by a factor of approximately 1000. Moreover, the highest bacterial counts were found when qPCR was used followed by PMA-qPCR and dilution plating method for the mixture of viable/dead cells. The difference in CFU or cells counts corresponded approximately to the amount of dead cells present in the mixture. In our study, the difference observed between qPCR and PMA-qPCR methods for viable cells may be explained by the presence of VBCN bacteria or cells with compromised membrane. This result indicates that PMA is able of penetrating the compromised cell membranes of dead cells without interferences of the matrix.

2.5. CONCLUSIONS

In this study we have established the optimal conditions for the specific detection and quantification of viable cells of the antagonist *P. agglomerans* CPA-2 by qPCR pre-treated with PMA. The procedure here outlined proved a specific detection of viable cells in both pure culture and artificially inoculated orange matrix. This PMA-qPCR method has the advantage to be specific, quantify viable bacteria and finally provide data on the VBNC fraction that is not detected by culture methods.

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CAPÍTULO III

DNA-based methodologies for quantification of live and dead cells in formulated biocontrol products based on agent *Pantoea agglomerans* CPA-2

Soto-Muñoz, L. Torres, R. Usall, J. Viñas, I. Solsona, C. Teixidó, N.

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ABSTRACT

The survival of *P. agglomerans* CPA-2 cells after dehydration by spray drying, fluidized bed drying and freeze drying was determined by microbiological and molecular methods, and these data were used to calculate treatment concentrations in efficacy trials. qPCR demonstrated an inability to differentiate between live and dead cells and thus overestimated the survival of CPA-2 in formulated products. Freeze-dried CPA-2 exhibited the greatest degree of survival. Furthermore, no significant differences in CPA-2 survival were observed as determined by dilution plating and PMA-qPCR after both the freeze drying and fluidized bed drying processes; however, an interesting significant difference was observed in the spray dried product. According our study, dilution plating may also not be a reliable tool for monitoring the survival of CPA-2 after spray drying. In contrast, the combination of PMA and qPCR enabled a quick and unequivocal methodology to enumerate viable and VBNC CPA-2 cells under stress-dried conditions.

Keywords: Spray-drying, freeze-drying, bed fluidized-drying, dilution plating, qPCR, PMA, survival.

3.1. INTRODUCTION

During the past decade, a whole range of microorganisms have been proposed as potential biocontrol agents (BCAs), including *Pantoea agglomerans* strain CPA-2, which is effective against the major postharvest pathogens present on pome and citrus fruits (Teixidó *et al.* 2001; Plaza *et al.* 2004; Cañamás *et al.* 2008a).

Dehydration is one of the best ways to formulate BCAs because these products can be handled using the normal distribution and storage channels (Rhodes, 1993). Different drying methods, such as freeze-drying, spray-drying and fluidized bed drying have been successfully tested for the formulation of some BCAs (Larena *et al.* 2003; Abadias *et al.* 2005; Guijarro *et al.* 2006; Yánez-Mendizábal *et al.* 2012). The main disadvantage of these processes is that they may affect a large number of cellular components, including DNA, RNA, the cytoplasmic membrane and the cell wall (Santivarangkna *et al.* 2008; Silva *et al.* 2011). In addition, the biological formulations should maintain an efficacy against plant pathogens that is similar to that of fresh cells (Teixidó *et al.* 2011).

To date, the viability of bacterial cells is most frequently assessed by plate counting. However, this method cannot provide the number of viable but non-culturable (VBNC) cells, i.e., cells that are stressed but retain metabolic activity or other essential cellular processes that contribute to viability. Consequently, the establishment of alternative viability assays that enable a fast and reproducible characterization of the heterogeneity of the total cell population with respect to different cellular processes is of great importance. Quantitative polymerase chain reaction (qPCR) has the potential to replace the conventional enumeration of BCAs (Spotts *et al.* 2009; Soto-Muñoz *et al.* 2014b). However, qPCR does not enable the distinction between DNA that arises from dead or living cells; therefore, DNA from non-viable cells contributes to the result (Soto-Muñoz *et al.* 2014b). To address this, a propidium monoazide (PMA) sample treatment step has been incorporated into the method (PMA-qPCR), ensuring the quantification of only viable cells with intact membranes. PMA can intercalate into DNA from dead cells with compromised membranes, and upon extensive visible light exposure, covalent binding to DNA occurs, and a suppression of PCR amplification results (Nogva *et al.* 2003; Nocker *et al.* 2006). PMA-qPCR has been successfully applied in studies of several food-borne

pathogens (Elizaquível *et al.* 2014) and probiotic bacteria, including *Lactobacillus acidophilus* and *Bifidobacterium animalis* (Kramer *et al.* 2009). Moreover, the optimal conditions of PMA treatment for CPA-2 have been established in previous studies (Soto-Muñoz *et al.* 2014a).

The aim of this work was to use dilution plating, qPCR and PMA-qPCR to assess the viability of formulated CPA-2, as obtained by spray drying, fluidized bed drying and freeze drying and to evaluate its use to calculate treatment concentrations in efficacy trials.

3.2. MATERIAL AND METHODS

3.2.1. Antagonist production

CPA-2 cultures were grown in a bench-top Biostat-A modular fermenter (Braun Biotech International, Melsungen, Germany) containing 5 L of an optimised growth medium based on sucrose/yeast extract medium (10 sucrose g/L, 5 yeast extract g/L, and 25 NaCl g/L) that was optimized by Cañamas *et al.* (2008b). After 24 h at 30 °C, 200 rev/min and 100 L/h of air feeding, CPA-2 cultures were harvested by centrifugation (9820×*g* for 10 min at 15 °C) and resuspended in potassium phosphate buffer (0.05 M, pH 6.5). The initial concentration of viable CPA-2 cells was determined by dilution plating, PMA-qPCR and qPCR, as described below.

3.2.2. *P. agglomerans* CPA-2 formulations

Cell pellets were used for the preparation of three CPA-2 formulations dried by: *(i) Spray drying:* CPA-2 cells were mixed with 10 % $MgSO_4$ and incubated at 150 rev min^{-1} for 30 min at room temperature. Subsequently, the suspension was spray-dried in a laboratory-scale spray dryer (SD-05; Lab Plant, Chelmsford, Esser, UK) under conditions reported by Costa *et al.* (2002a); *(ii) Freeze drying:* CPA-2 cells were resuspended in deionised water containing 10 % sucrose (as a protectant), frozen at -20 °C overnight and freeze-dried (Cryodos, Telstar SA, Terrasa, Catalonia, Spain) at 1 Pa and -45 °C for 24 h (Costa *et al.* 2000); and *(iii) Fluidized bed drying:* the cell pellet was mixed with potato starch (carrier) in a proportion of 60:40 (carrier:bacterial paste). The mixture was homogenized and extruded into pellets of 3-5 mm length and 1 mm diameter. These pellets were dried in a fluid bed-dryer 350s (Burkard Manufacturing Co. Ltd, Hertfordshire, UK) at an inlet air temperature of 40 °C for 20 min (Usall *et al.* 2010). To determine the amount of dry matter in the initial suspensions of fresh CPA-2 cells and in the final formulated products, duplicate samples of 5 mL and 0.5 g, respectively, were dried in a convection oven at 105 °C for 24 h.

3.2.3. Survival determination

Three samples of each product obtained by spray drying (0.25 g), fluidized bed drying (0.05 g) or freeze drying (0.5 g) were rehydrated in 5mL of 10 % non-fat skimmed milk (NFSM) or water (Costa *et al.* 2002b).

The cell concentrations were determined by dilution plating, PMA-qPCR and qPCR. *(i)* To quantify cells by dilution plating, ten-fold dilutions were made in a phosphate buffer solution, dilutions were plated on NYDA media, and they were incubated at 30 °C for 24 h. *(ii)* To quantify cells by PMA-qPCR, 100 µL samples of spray dried and fluidized bed dried CPA-2 or 10 µL samples of freeze dried CPA-2, previously rehydrated, were centrifuged (9727×*g* for 20 min), and cell pellets were suspended in 500 µL of phosphate buffer solution and treated with PMA (Biotum, Hayward, USA), as described by Soto-Muñoz *et al.* (2014a). Each replicate was treated in triplicate. After PMA treatment, cells were centrifuged (9727×*g* for 20 min) prior to DNA extraction, according to the method described by Crespo-Sempere *et al.* (2013), and cells were evaluated by qPCR under conditions defined by Soto-Muñoz *et al.* (2014b). *(iii)* Cells were quantified by qPCR without PMA treatment as described by Soto-Muñoz *et al.* (2014a). A negative control was included using water instead of genomic DNA, and all reactions were performed in triplicate. An absolute quantification of CPA-2 was achieved by constructing a standard curve using genomic CPA-2 DNA extracted from a range of bacterial cell concentrations (from 3×10^3 to 3×10^7 cells/mL). Amplification efficiency was calculated from the slope of the standard curve: $E=10^{-1/slope}$; % Efficiency = (E–1) × 100.

3.2.4. Biocontrol efficacy trial

Viable CPA-2 cells that were formulated by spray drying and quantified by dilution plating and PMA-qPCR were tested on oranges (cv. *Valencia Late*) for their efficacy against *P. digitatum*. A *P. digitatum* inoculum adjusted to 10^5 conidia/mL was prepared from a 10-day-old culture grown on PDA. Experimental treatments were prepared from spray dried CPA-2 formulations that were rehydrated with NFSM or water, and the efficacy was compared to that of fresh cells and rehydration media without CPA-2 cells as a control. Fresh cells were prepared following the methodology described by Teixidó *et al.* (2001). All treatments were prepared at 10^8 CFU or cells/mL, and the concentration of spray-dried CPA-2 was determined by dilution plating and PMA-qPCR.

Oranges were wounded by making two injuries with a nail (3 mm wide and 3 mm deep) in one side of the fruit, and they were then inoculated with 15 µL of *P. digitatum*. After air-drying, 15 µL of each CPA-2 treatment (a fresh CPA-2 suspension, spray-dried suspensions quantified both by dilution plating and PMA-qPCR, and rehydration media) was applied to the wounds. Fruits were incubated at 20 °C and 85 % relative humidity

(RH) for 4 days, and the decay incidence and severity were measured. Fruit trials were carried out in four replicates using five fruits per replicate.

3.2.5. Statistical analysis

The survival of *P. agglomerans* CPA-2 from the three methods was calculated as $\log_{10}$ CFU or cells per g of dry weight (g·dw). The significance of difference between the survival of CPA-2 formulations, the incidence and severity were analysed using analysis of variance (ANOVA) with JMP®8 statistical software (SAS Institute, Cary, NC, USA). Statistical significance was judged at the level $p<0.05$.When the analysis was statistically significant, Student's *t*-test was used for the separation of means.

3.3. RESULTS AND DISCUSSION

The standard curve showed a strong linear relationship (0.977) among the DNA extracted from different CPA-2 concentrations ($5.2x10^4$ to $5.2x10^8$ cells dry/weight per reaction) and *Cq*-values (Figure 1). When *Cq*-values were plotted against cell concentrations, a slope of -3.248 was obtained, corresponding to an efficiency of 2.03 (103 %).

In general, the bacterial enumeration of CPA-2 was higher when NFSM was used as the rehydrating medium than when water was used, but this difference was not statistically significant ($p < 0.05$) (data not shown). Therefore, quantification data for the different drying methods could be pooled for the statistical analysis. In other studies on rehydrating freeze-dried *Candida sake* cells, cell viability increased two fold after rehydration with NFSM compared with water (Abadias *et al.* 2001). Costa *et al.* (2002a; 2002b) also found that NFSM was the best rehydration medium tested in spray- or freeze-dried *P. agglomerans* due to ability of this complex medium to repair damaged cells and improve the final recovery.

In our study, CPA-2 showed the greatest survival when dehydrated by freeze drying, followed by fluidized bed drying and spray drying (Figure 2). The minimal amount of freeze-dried CPA-2 cell death could be due to the use of sucrose as a protective substance. Sugars, especially disaccharides, seem to play a role in the stabilization of dry biological membranes (Crowe *et al.* 1984). Costa *et al.* (2000) demonstrated that the viability of *P. agglomerans* CPA-2 after freeze drying decreased when the concentration of sucrose was reduced.

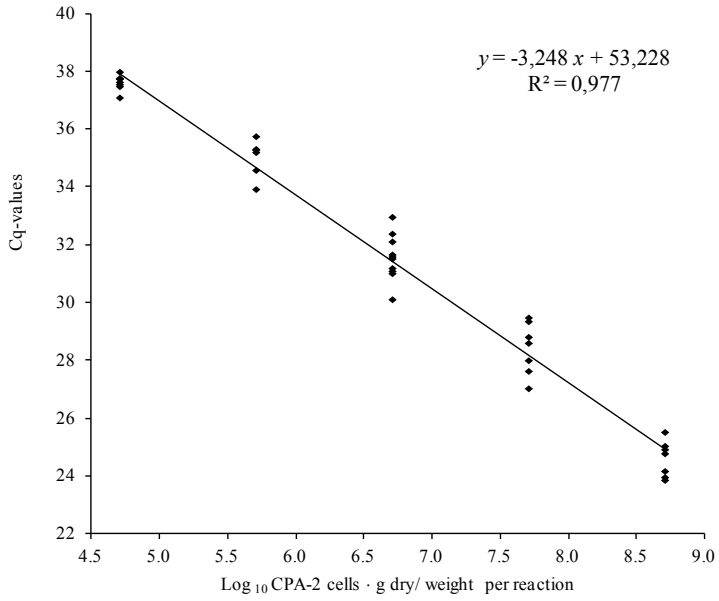

Figure 1. Real time PCR amplification plots for reaction with genomic DNA templates of bacteria suspension (plots 1-5: ranged from 10^4 to 10^8 cells per reaction). DNA templates were extracted from serial diluted fresh bacterial cells.

The suitability of dilution plating, qPCR and PMA-qPCR for reliably determining CPA-2 cell survival under stress-dried conditions was examined. The results showed a negligible difference (below 0.23 $\log_{10}$ CFU or cells/g·dw) in the survival of freeze-dried CPA-2 cells among the three evaluated methods of quantification. Similar results were previously obtained by Soto-Muñoz *et al.* (2014a) on CPA-2 cells subjected to freeze drying. In addition, our results showed a similar pattern to those observed in a study determining the survival of *Lactobacillus acidophilus* and *Bifidobacterium animalis* ssp. *lactic* cells after freeze dying (Kramer *et al.* 2009). According to the authors and accounting for minimal differences observed in this work, any method of quantification tested here could be used to determine the survival of freeze-dried CPA-2 cells.

CPA-2 cells that were dehydrated by the fluidized bed drying process showed a reduction in survival of 1.05 $\log_{10}$ cells/g·dw, as determined by qPCR, and the reduction was 1.73 and 1.81 $\log_{10}$ CFU or cells/g·dw as determined by dilution plating and PMA-qPCR, respectively. The similar values obtained by dilution plating and PMA-qPCR indicate a loss of cell cultivability and of membrane integrity. Therefore, most of the reduction in survival can most likely be attributed to membrane permeabilization during the drying process. It is generally accepted that the loss of membrane integrity in a bacterial cell reflects the absence of reproductive growth and metabolic activity,

eventually leading to cell death (Nebe-von Caron and Badley, 1996). Bensche *et al.* (2014) observed a similar viability pattern with fluidized bed dried *Lactobacillus plantarum* cells whose viability was determined by dilution plating; in this study, flow cytometry was used with SYBR Green and propidium iodide to assess membrane integrity. The loss of membrane integrity can be caused by dehydration and thermal or oxidative stress (Santivarangkna *et al.* 2008; Bensch *et al.* 2014). In addition to dehydration, oxidative stress might have also been involved in membrane damage during drying, e.g., by oxygen radicals or by reactive oxygen species (ROS), resulting in lipid peroxidation (Shafiei *et al.* 2014).

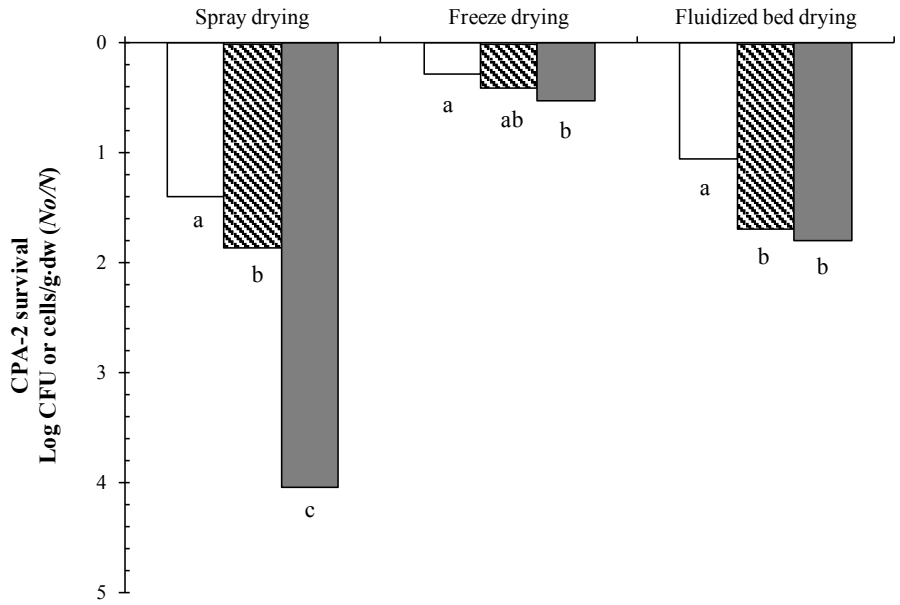

Figure 2. Survival of *Pantoea agglomerans* spray dried, freeze-dried or fluidized bed dried quantified by qPCR (□), PMA-qPCR (▨), and dilution plating (■). Levels of viability after drying process were expressed as logarithmic value of survival fraction $\log_{10}$ (*N/No*) (*No*: CFU or cells g dry/weight before dried-fresh cells; *N*: log CFU or cells per g of dry weight (g·dw)). Each bar represents the average of three independent replications of two assays and different letter indicate significant differences between drying method ($p<0.05$) according the Student's *t*-test.

In this study, significant differences on the survival of spray-dried CPA-2 cells were observed among the three quantification methods. A difference of 0.48 $\log_{10}$ cells was observed between PMA-qPCR and qPCR. Therefore, it seems that a fraction of spray-dried CPA-2 cells could have obtained membrane damage as a result of the spray drying process. A significant difference of 2.17 $\log_{10}$ CFU or cells/g·dw was observed

between the PMA-qPCR and dilution plating methods, the latter method underestimating the number of viable cells. Several studies have reported that the high temperatures used in spray drying can caused damage to the cell wall and membrane (Ananta *et al.* 2005; Fernández-Sandoval *et al.* 2012). In addition to membrane damage, the high temperature also induces some deteriorative reactions, such as protein oxidation, lipidation and glycation, which may cause further protein modifications and protein insolubility (Peighambardoust *et al.* 2011). These modifications can partly account for the changes in cell viability. It can also be deduced that even moderate carbonylation of some critical cellular proteins (such as ribosomal proteins) may lead to VBNC cell formation or bacterial death (Shafiei *et al.* 2014). These facts might explain how a portion of spray-dried CPA-2 cells were not detected by dilution plating or PMA-qPCR. Furthermore, Fittipaldi *et al.* (2012) reported that the quantification of live cells by PMA-PCR in the presence of high levels of dead cells can affect the efficiency of the method.

In addition, the survival of CPA-2 cells after both fluidized bed drying and spray drying was overestimated by qPCR. Therefore, the DNA from CPA-2 cells that were lysed during drying was quantified by qPCR, showing the unreliability of the method for the quantification of intact bacteria mixed with large numbers of injured and dead cells. Similar results were observed in other studies and demonstrate the inability of qPCR to differentiate between live and dead cells as the main drawback of this method (Reichert-Schwillinsky *et al.* 2009; Morales *et al.* 2013).

3.3.1. Biocontrol efficacy trial

The effectiveness of spray dried CPA-2 against *P. digitatum* in *Valencia Late* oranges is shown in Figure 3 under various conditions: CPA-2 rehydrated with water or NFSM at $1x10^8$ CFU or cells/mL, determined by dilution plating or PMA-qPCR, and compared with fresh CPA-2 cells and untreated cells that were rehydrated with water or NFSM. After 3 days of incubation, a significant difference was observed between the rehydration media used both in the control samples and biocontrol treatments: the NFSM control showed the lowest values of incidence and severity, 75 % and 1.42 cm, respectively, while water controls showed values of 95 % of incidence and 1.83 cm of severity (Figure 3A). However, when the incubation time was increased to 4 days, no differences were observed: NFSM and water controls showed 100 % of incidence and severities of 4.63 and 4.01 cm, respectively (Figure 3B); therefore, the use of NFSM did not affect the growth of *P. digitatum* on the orange surface. Moreover, no significant differences in the incidence and severity of infection were observed between spray dried CPA-2 that was adjusted by dilution plating and that which was adjusted by PMA-qPCR after 3 and 4 days of incubation. After 3 days of incubation (Figure 3A), spray dried cells rehydrated

with water showed a reduction of incidence of 70 and 62 % between those quantified by dilution plating and PMA-qPCR, respectively, compared with the control.

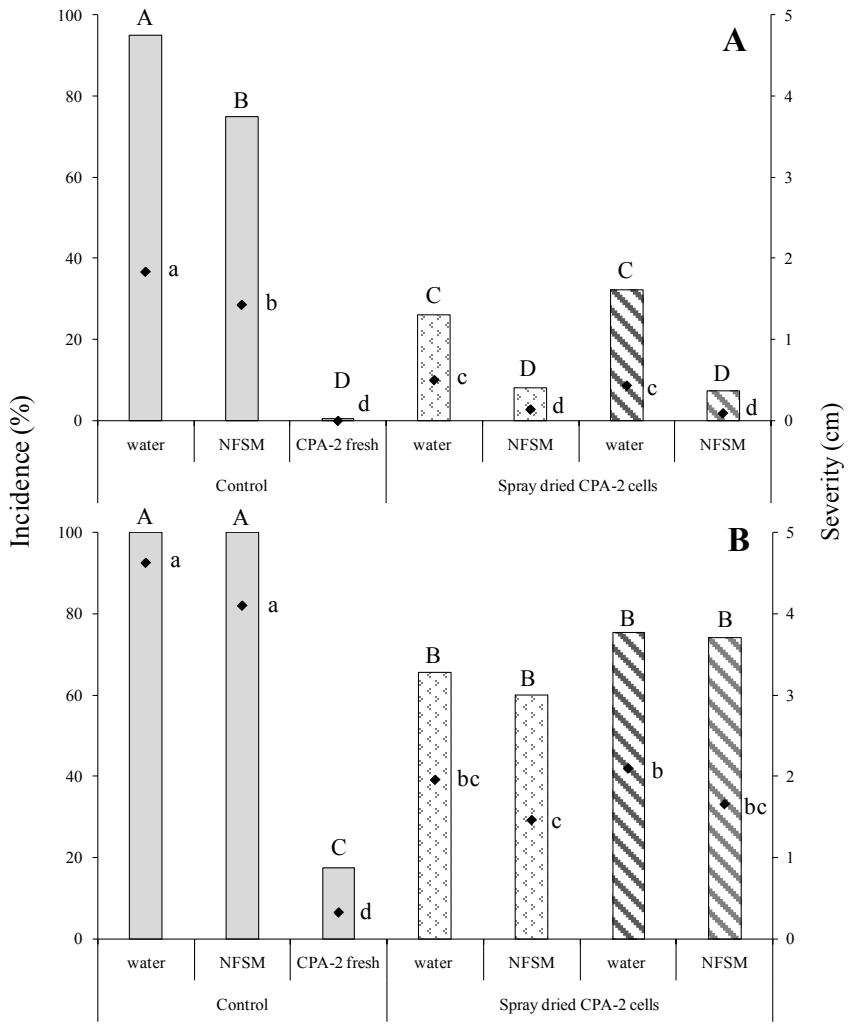

Figure 3. Incidence (bars) and severity (solid diamond shape) in *Valencia Late* oranges inoculated with *Penicillum digitatum* and then treated with spray dried CPA-2 cells rehydrated with non-fat skim milk (NFSM) or water and adjusted at 1x10^8 CFU/mL by dilution plating (⬚) and PMA-qPCR (◪). Rehydrated spray dried CPA-2 cells treatments were compared with CPA-2 fresh cells (24 h-old culture without spray-drying) at 1x10^8 CFU/mL and untreated controls with water or NFSM (■). Evaluation of disease incidence and lesion diameter was performed 3 days (A) and 4 days (B) after inoculation. Values are means of 5 fruits x 4 replicates. Mean values with same upper case (incidence) or lower-case letters (severity) are no significantly different (p=0.05) according to Student's t-test.

Spray dried cells rehydrated with NFSM showed a similar effect on *P. digitatum* to that of fresh cells. Unlike the results obtained after 3 days of incubation, the efficacy of spray dried cells was significantly lower after 4 days of incubation than that obtained with fresh cells (Figure 3B). In spite of the difference between the concentrations of viable cells that is described by PMA-qPCR and dilution plating, which is approximately 1.70 $\log_{10}$ CFUs for the spray dried CPA-2 cells, samples from both methods maintained the same degree of control against *P. digitatum*. The mode of action used by *P. agglomerans* CPA-2 is nutrient competition or site exclusion (Poppe *et al.* 2003), Therefore, it is possible that damaged spray-dried cells are metabolically active and could able be to colonize the orange wound and prevent *P. digitatum* development. Thus, the use of dilution plating may not be a reliable tool for monitoring the survival of CPA-2 cells.

3.4. CONCLUSIONS

This study has shown that the enumeration method deeply influences the quantification studies. Therefore, it should be carefully considered depending on the physiological state bacteria and the dehydration process used for its formulation. The dilution plating method is not a reliable enumeration technique for high-stress conditions, such as in spray drying. The use of PMA overcomes the inability of the qPCR method to differentiate between alive and dead cells. The combination of PMA-and species- specific qPCR in this study allowed a quick and unequivocal way of enumeration of viable and VBCN of *P. agglomerans* CPA-2 cells under stress-dried conditions. Studies for verifying damage cell of this BCA after drying process will need to be addressed in future studies.

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CAPÍTULO IV

Environmental monitoring of the biocontrol agent *Pantoea agglomerans* CPA-2 applied on citrus fruit in preharvest

Soto-Muñoz, L. Torres, R. Usall, J. Viñas, I. Dashevskaya, S. Teixidó, N.

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ABSTRACT

The aim of this study aim was to evaluate the environmental fate and behavior of the biocontrol product based in *Pantoea agglomerans* CPA-2 cells after its release under field conditions. Three different methods, dilution plating, PMA-qPCR and qPCR were used to track CPA-2 population. Populations quantified by qPCR were statistically different compared with PMA-qPCR and dilution plating methods. In addition, the spray dispersion of CPA-2 treatment was evaluated using water-sensitive papers. A lack dispersion of CPA-2 treatment was observed at distances of 2.5±0.5 m. The presence and persistence of CPA-2 in the environment were monitored in different areas, by dilution plating and confirmed by conventional PCR. The results showed that CPA-2 can survive but not proliferate on ground, weed and leaves. The persistence on inert surfaces, such as, motorized backpack sprayer, gloves and working clothes was lower than 7 days. In conclusion, PMA-qPCR was a potential tool to quickly and specifically monitor viable populations of CPA-2 and it gave valuable information on population behavior. Moreover, the results demonstrate that CPA-2 would not present a risk for the environment due to its low establishment, survival and dispersion.

Keywords: Orange, molecular marker, qPCR, persistence, viable cells, PMA.

4.1. INTRODUCTION

The control of fungal diseases in fruits and vegetables is still mainly based on the use of synthetic fungicides, although the demand of free chemical residues and the emergence of fungicide-resistant pathogen strains are constantly increasing. Biological control using microbial antagonists has attracted much interest as an alternative to chemical products (Viñas *et al.* 1998; Droby *et al.* 2009). In this context, the antagonistic activity of *Pantoea agglomerans* strain CPA-2 against blue and green moulds in citrus fruit under pre- and postharvest conditions has been clearly demonstrated (Teixidó *et al.* 2001; Plaza *et al.* 2004; Torres *et al.* 2007; Cañamás *et al.* 2008; Usall *et al.* 2008) and the formulated product, is now available although it is not being commercialized (Torres *et al.* 2014).

The registration of a biocontrol product is required before any commercial use, as a usual food safety procedure. In Europe, the situation is more complex than in other parts of world, and until recently, registration has been difficult or even no possible to obtain for biological control agents (BCAs) (Droby *et al.* 2009). The registration for applying plant protection products are regulated by EC Regulation 1107/2009 which also defines time frames for the registration process. In particular, Annex II of the directive established issues that need to be addressed, including the estimation of the fate and distribution of the microorganism in the environment and its impact on non-target species. To conclude with this requirement of the directive, a perfect identification of the BCA at the species and strain level is an absolute necessity (Alabouvette and Cordier, 2011).

The use of molecular markers to distinguish BCA strains from other epiphytic microorganism present in the environment offers much promise for the rapid identification of this specific bacterial strain. PCR-based methods designer using SCAR markers have been developed to specifically detect and monitor several BCAs (Schena *et al.* 2002; De Clercq *et al.* 2003; El Hamouchi *et al.* 2008; Edel-Hermann *et al.* 2011), including *P. agglomerans* CPA-2 strain (Nunes *et al.* 2008).

Conventional PCR is a sensitive technique that can detect a single target molecule and also rapid and versatile. However, PCR is unreliable for quantitative analysis (Schena *et al.* 2013). In contrast, quantitative real-time PCR (qPCR) offers the possibility to quantify and accurately detect specific BCAs and monitor their population dynamics over a period of time (Sanzani *et al.* 2014). In a previous work, a qPCR method has been developed to quantify *P. agglomerans* CPA-2 on apple surfaces after postharvest application (Soto-Muñoz *et al.* 2014). This method has the advantage to detect cultivable and viable but noculturable (VBNC) cells, at short periods of storage time. Nevertheless, during a long storage time, qPCR showed an overestimation of the population of CPA-2, due to inability to differentiate viable and nonviable cells.

In this sense, a new methodology based on cell membrane integrity as a well-accepted criterion for distinguishing viable from dead cells and using the specificity and sensitivity of qPCR has been developed (Nocker *et al.* 2006; Fittipaldi *et al.* 2012). This methodology takes advantage of the fact that a nucleic acid intercalating dye, the propidium monoazide (PMA) selectively penetrates permeable cells membranes of dead bacteria and covalently link, to DNA by photolysis under bright visible light. This irreversible DNA modification results in a suppression of PCR amplification (Nogva *et al.* 2003; Nocker *et al.* 2006). PMA treatment combined with qPCR (PMA-qPCR) has been successfully tested in simple matrices such as pure and mixed cultures on food microorganisms such as *Listeria monocytogenes* (Pan and Breidt, 2007), *Escherichia coli* O157:H7 (Dinu and Bach, 2013) and *Campylobacter jejuni* (Josefsen *et al.* 2010). Not only PMA has been applied to discriminate between dead and live bacteria; it has also been used to identify viable yeasts (Andorra *et al.* 2010) and fungi (Crespo-Sempere *et al.* 2013). The optimal conditions of PMA treatment for the strain CPA-2 identification on orange fruit has been established in previous studies (Soto-Muñoz *et al.* 2014). Thus, PMA-qPCR technique could be a useful tool to quickly and specifically monitor population of *P. agglomerans* CPA-2 viable on citrus under field conditions.

The main objectives of this study were *(i)* to quantify the populations of *P. agglomerans* CPA-2 on orange surface, previously treated in preharvest conditions, by dilution plating, PMA-qPCR and qPCR *(ii)* to evaluate spray dispersion treatment applied of CPA-2, and *(iii)* to determine the environmental distribution and persistence of CPA-2 cells at field conditions.

4.2. MATERIAL AND METHODS

4.2.1. The antagonist

The CPA-2 strain of *P. agglomerans* used in this study was obtained from IRTA Centre in Lleida (Catalonia, Spain). This strain was isolated from Golden Delicious apples and is currently deposited at the Spanish Collection of Type Culture (CECT, University of Valencia, Valencia, Spain), as CECT-4920. Stock cultures were stored long-term at −80 °C in cryogenic vials and subcultured on nutrient yeast dextrose agar (NYDA: 8 g/L nutrient broth, 5 g/L yeast extract, 10 g/L dextrose and 20 g/L agar). The activated culture was maintained on NYDA at 30 °C for 24 h and transferred to potassium phosphate buffer (pH 6.5) (0.2 mol/L KH_2PO_4, 70 mL; 0.2 mol/L K_2HPO_4, 30 mL and deionised water, 300 mL) to obtain a cell suspension. This suspension was used as an inoculum for biomass production in a fermentation system. Osmotically adapted cells grown in a liquid medium (5 g/L yeast extract, 10 g/L sucrose and 25 g/L NaCl), as previously described Cañamás *et al.* (2008) were used in all studies. An appropriate volume of inoculum was added to 5 L of the liquid medium adjusted to $1x10^6$ CFU/mL. Cultures were grown in a 5 L bench-top BIOSTAT-A fermenter (Braun Biotech International, Melsungen, Germany) at 30 °C with 300 rpm agitation and 100 L/h aeration. Cultures were harvested at stationary phase (24 h) by centrifugation at 9820 *g* for 10 min at 15 °C in an Avanti™ J-25 centrifuge (Beckman, Palo Alto, Ca, USA). Cell pellets were resuspended in deionised water containing 10 % sucrose, frozen at -20 °C overnight and freeze-dried (Cryodos, Telstar SA, Terrasa, Catalonia, Spain) at 1 Pa and -45 °C for 24 h as described by Costa *et al.* (2000). These lyophilised cells were packaged in a white high-density polyethylene bottle at non-vacuum atmosphere (Torres *et al.* 2014).

4.2.2. Field treatment

Field experiment was carried out in a commercial orchard located in the Montsià area, South of Tarragona, Catalonia, Spain. The formulated and packaged cells were applied on Valencia cultivar oranges 16 days before harvest, on May of 2012. Weather variables such as temperature and relative humidity (RH) were collected during the field treatment using an electronic sensor (data-logger) positioned in the crop canopy of orchard at a height of 1 m.

CPA-2 treatment was prepared by adding an adequate volume of formulated cells, previously rehydrated in 1 % non-fat skim milk, into 30 L of water in order to obtain a cell suspension at a concentration of 2×10^8 CFU/mL (Cañamás *et al.* 2008). In addition, 50 g/L of Fungicover (BioDúrcal S.L.) was added to the cell suspensions. The additive Fungicover is a commercial formulation of derivatives of fatty acids in an

aqueous-alcoholic solution and previous research showed that this additive improved *P. agglomerans* survival on orange under field conditions (Cañamás *et al.* 2008). The cell suspension treatment was sprayed at 15 atm with a motorized backpack sprayer (nozzle size, 1 mm; Honda model WJR 2225, Germany) at a rate of 400 L/ha until near run-off on orange trees.

The experimental design of each plot consisted of treated and non-treated trees. Four replicates of two consecutive trees per replicate were directly sprayed with CPA-2 treatment (designated as "treated"). Each replicate was separated by two untreated trees (designated as "non-treated"). Treated and non-treated trees within a plot were separated by five trees. Treated trees were used to quantify CPA-2 populations on surface orange fruits. Treated and non-treated trees were also used to evaluate spray dispersion of treatment and the environmental distribution and persistence of CPA-2 strain in the field.

4.2.3. Quantification of *P. agglomerans* CPA-2 on orange surface

Populations of *P. agglomerans* CPA-2 on orange surface were determined by three quantitative methods: *(i)* dilution plating to obtain the active-culturable population *(ii)* PMA-qPCR technique to obtain viable cell counts, and *(iii)* qPCR to obtain the total cell count. Five fruits per replicate were sampled and four replications were collected separately from both the sun-exposed (designated as "OUT-fruits") and shaded canopy position (designated as "INS-fruits"). The recovery of CPA-2 cells from oranges surfaces was performed according to the method described by Torres *et al.* (2012), with minor modifications. Briefly, eight pieces of peel (16 mm diameter) were removed from each fruit. Peel samples were mixed with 20 mL of 0.05 mol/L phosphate buffer solutions and pummelled in a Stomacher 400 set at normal speed for 90 s and the resulting mixture was filtered through a 11 µm porous filter (Whatman® International, Maidstone England). The sample suspension was used for further quantification of CPA-2. To quantify by dilution plating, ten-fold dilutions were made in phosphate buffer solution and plated on NYDA media. Each suspension was analyzed twice by dilution plating. The colonies were counted after incubation of plates at 30 °C for 24 h. To quantify by PMA-qPCR and qPCR methods, as described in next sections was used.

4.2.3.1. PMA treatment

Treatment of samples with PMA (phenanthridium, 3-amino-8-azido-5-[3- (diethylmethylammonio) propyl]-6-phenyl dichloride; Biotum, Hayward, USA) was performed based on optimization previously described by Soto-Muñoz *et al.* (2014). Briefly, PMA stock of 20 mmol/L in water was added to 4 mL of sample suspension to obtain a final PMA concentration of 30 µM. Each PMA-treated sample was incubated for

20 min in the dark and then exposed to LEDs light source during 30 min. Afterward photo-induced cross-linking, cells were centrifuged (Hettich Mikro 22R, Germany) at 9727 *g* for 20 min at 4 °C prior to DNA extraction, followed by qPCR quantification.

4.2.3.2. DNA extraction and qPCR

Genomic DNA of *P. agglomerans* CPA-2 was extracted using Lyse-N-Go PCR Reagent (Thermo Scientific, Rockford, IL, USA) following the manufacturer's instructions. The amount and purity of DNA samples were determined spectrophotometrically (NanoDrop ND-1000; NanoDrop Technologies, Wilmington, DE) and DNA integrity was evaluated by electrophoresis on 1.5 % agarose gel run at 70 V for 90 min with TEB buffer and stained with 0.5 mg/µL ethidium bromide. The ABI-7500 qPCR detection system (Applied Biosystem Division, Perkin-Elmer Co. Foster City, CA, USA) was used to quantify genomic CPA-2 DNA. PCR reactions were performed in a final volume of 25 µl containing 1× PCR *Taqman* Universal PCR Master Mix (Applied Biosystem, USA), 300 nmol/L of SP_2-forward primer (CTTAAAGCGCAGGGAAGCCGGTCAG), 300 nmol/L of SP_2-reverse primer (GAGCCGGCTCAGGGAAACCGGTC), 200 nmol/L of *Taqman* MGB probe (FAM-TCCATGGATGGCTTAAG-MGB) defined in a previous work (Soto-Muñoz *et al.* 2014) and 4 µl of DNA extracted with and without use of PMA treatment diluted 10-fold. Cycling conditions used were: 2 min at 50 °C, 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 59 °C. In all cases, amplification of duplicate DNA samples spiked with 10 ng of CPA-2 DNA was used to verify that negative results were not due to polymerase inhibition. Two negative controls were included: *(i)* DNA extracted from untreated fruits and *(ii)* using water instead of genomic DNA. Data acquisition and analysis were performed using SDS software version 1.3.1 (Applied Biosystems) according to the manufacturer's instructions. The quantification cycles (*Cq*) values were automatically calculated for each reaction by the SDS software. Any sample that reached a fluorescence value exceeding the fluorescence threshold value was considered positive. All reactions were performed in triplicate.

A standard quantification curve was prepared by adding a range of bacterial cell concentrations (from 1.5×10^2 to 1.5×10^6 cells/cm^2) from orange suspensions. These orange suspensions were obtained from 40 pieces of peel (16 mm diameter) of five untreated fruits in 20 mL of 0.05 mol/L phosphate buffer solution, pummelled in a Stomacher 400 set at normal speed for 90 s. DNA extraction was done as described above. The *Cq* values obtained were used to plot a standard curve by assigning the corresponding concentration values. Standard curve obtained was $y = -3.08x + 49.68$; $R^2 = 0.99$.

4.2.4. Evaluation of spray dispersion of *P. agglomerans* CPA-2 in preharvest applications

The spray dispersion of CPA-2 treatment at field applications was evaluated with water-sensitive papers (WSP) (Figure 1). The WSP were fixed through a nail in *(i)* the leaves on the "outer" canopy of the tree and the leaves on the "inner" canopy of the tree. Each position, papers had one position upward and one downward facing paper (i.e. outer-upward, outer-downward, inner-upward, and inner-downward), both in the treated and non-treated trees, *(ii)* on ground around of treated and non-treated trees (four papers by replication), at 1/2 or 2/3 tree of distance between both treated and non-treated trees. WSP were removed as soon as they were dry and then stored in a zip-lock bag, until their analysis. Images of each WSP were digitatized using a Hewlett-Packard scanner. The percentage spray cover on each paper was estimated by image analysis using ImageJ Software (ImageJ, US National Institutes of Health, available in the public domain on the Internet at http://rsb.info.nih.gov/ij/).

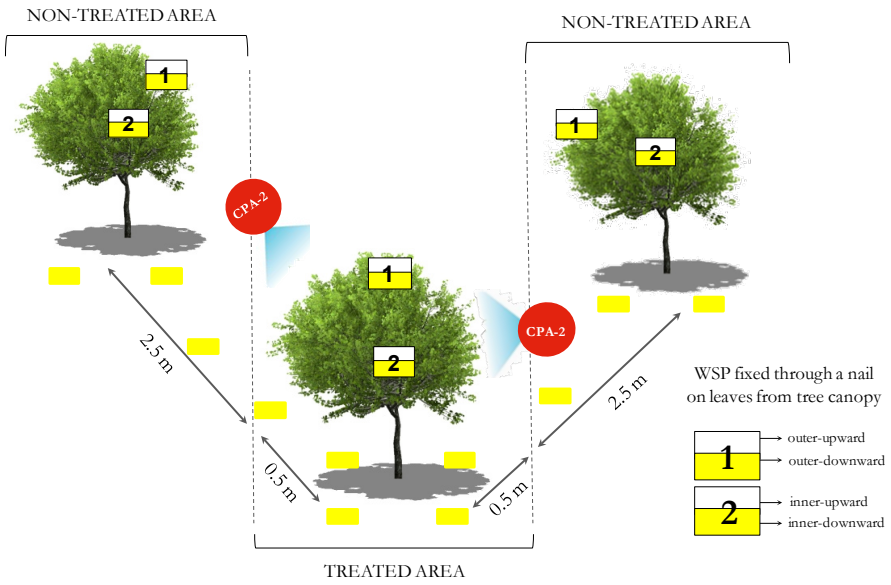

Figure 1. Plan of experimental and sampling layout of treatment spray *P. agglomerans* CPA-2 under field conditions

4.2.5. Study of environmental distribution and persistence of *P. agglomerans* CPA-2 in preharvest applications

Three sampling sites were selected to monitor CPA-2 on different surfaces and environments: *(i)* treated area, *(ii)* non-treated area and *(iii)* motorized backpack sprayer, gloves and working clothes worn by handlers.

The sample points in the treated area were: *(i)* weed and ground around treated trees, and *(ii)* leaves and fruits of treated trees. Weed and ground samples were collected around treated trees in two different points of each replicate tree. Leaf samples consisted of 10 leaves collected from the top, middle, and bottom of both the sun-exposed (designated as "OUT-leaves") and shaded canopy position (designated as "INS-leaves") of each replicate tree. All samples were transported to the laboratory at cold conditions. Afterwards, one gram of weed or ground sample were mixed with 5 mL of 0.05 mol/L phosphate buffer solution in a sterile stomacher filter bag. Leaf sample consisted of two pieces of 16 mm diameter of each leaf mixed with 10 mL of 0.05 mol/L phosphate buffer solution in a sterile stomacher filter bag. Samples were pummelled in a Stomacher (Seward, London, UK) set at normal speed for 90 s. Serial 10-fold of suspension samples were made and plated on NYDA media. Each suspension was analyzed twice by dilution plating. The colonies were counted after incubation of plates at 30 °C for 24 h. The sampling times were: just after treatment application (day 0), and 1, 3, 7, 16 and 21 days after treatment. To distinguish *P. agglomerans* CPA-2 from other microorganisms, yellow colonies with phenotypic characteristics similar to *P. agglomerans* were randomly selected from plates of all sampling times and evaluated for conventional PCR test. The number of colonies selected was maximum of 25 colonies per plate, but always at least 10 % of all colonies per plate.

The sample points in the non-treated area were: the air, weed around non-treated trees, and leaves and fruits of non-treated trees. The air was sampled according to the gravimetric method. Eight petri dishes (four replicates) containing NYDA medium supplemented with imazalil (imazalil sulphate 99 %, 0.5 g/L) were equidistantly placed around each plot of treated trees and at 1/2 or 2/3 tree of distance between treated and non-treated trees. The petri dishes were left open for 2 min, just after treatment.

The weed, leaves and fruits of non-treated trees were sampled after treatment (day 0), and 1, 3, 7, 16 and 21 days after treatment. The sampling method used was contact plating method using Rodac plates containing NYDA medium with 0.5 g/L imazalil, by contact between the culture medium and the surface, with slight pressure to get adhered microorganisms to the medium. For each sampling, eight Rodac plates (replicates) were used on each sample point.

Finally, the motorized backpack sprayer, the gloves and working clothes worn by handlers also were sampled to determinate the presence and persistence of CPA-2. The motorized backpack sprayer was sampled from 0 to 5 days and five Rodac plates were used in each sample time. The gloves and working clothes were sampled from 0 at 2 days and from 0 to 5 days after treatment, respectively. For each sampling, two Rodac plates were used on each glove pair, and nine Rodac plates were used on the working clothes.

All Rodac and NYDA plates used at each sampling were incubated at 30 °C for 48 h. All yellow colonies with phenotypic characteristics similar to *P. agglomerans* were selected from plates of all sampling days and identified by conventional PCR test.

4.2.5.1. Conventional PCR

To identify *P. agglomerans* CPA-2 by conventional PCR was used the primer pair PAGA1 and PAGB1 described previously by Nunes *et al.* (2008). This primer pair amplify two different fragments: a first SCAR marker (720 bp) that is specific to strain CPA-2 and the second (270 bp) is specific to *P. agglomerans* species. Fifty microlitres of amplification reaction was prepared using 20 ng of each genomic DNA, 0.5 units of *Taq* DNA polymerase in 1 × reaction buffer (Invitrogen Life Technologies, Carlsbad, CA, USA), 0.2 mmol/L dNTPs and 0.4 mmol/L of each SCAR primer. DNA amplification was carried out in a Peltier thermal cycler (GeneAmp PCR System 2700, Applied Biosystems) with the following programme: an initial denaturation step at 95 °C for 5 min followed by 40 cycles of 95 °C for 30 s, 59 °C for 45 s and elongation at 72 °C for 90 s followed by a final extension step at 72 °C for 10 min. The PCR products were analyzed by electrophoresis as described above. DNA standard (100 bp DNA Ladder, Invitrogen Life Technologies, Carlsbad, CA, USA) was used as molecular size marker.

4.2.6. Statistical analysis

The *P. agglomerans* CPA-2 population level from three quantitative methods was calculated as CFU or cells/cm^2 of the orange surface with its standard error and a 95 % confidence interval. Population size (CFU or cells/cm^2) was log transformed to improve homogeneity of variances. The percentage spray cover was analyzed using analysis of variance (ANOVA) with JMP® 8 statistical software (SAS Institute, Cary, NC, USA). Statistical significance was judged at the level $P<0.05$. When the analysis was statistically significant, the Student's *t*-test for separation of means was used.

4.3. RESULTS

4.3.1. Environmental data

The weather conditions during the experimental period, daily maximum temperatures values ranged between 20 and 31 °C. The daily minimum temperatures were between 11 and 17.5 °C. Daily maximum values of RH ranged from 80 to 100 %. However, during the first 4 and 6 days after treatment it declined from around level of 51 and 60 %. With regard to the daily minimum observed values RH ranged 24 to 63 %. A rainfall of 1 and 8 mm, were observed in days 7 and 21, respectively (data not shown).

4.3.2. Quantification of *P. agglomerans* on orange surface

The quantification of *P. agglomerans* CPA-2 on OUT-fruits and INS-fruits of treated trees was evaluated by three methods, dilution plating, PMA-qPCR and qPCR (Figure 2). It was observed significant interaction among fruit position (fruits exposed and shaded canopy position) and population present, thus data were analyzed separately. The amount of *P. agglomerans* CPA-2 quantified on OUT-fruits (Figure 2A) by dilution plating was approximately 4.50 $\log_{10}$ CFU/cm^2 at time 0 and decreased drastically during the first 3 days until 1.84 $\log_{10}$ CFU/cm^2. Then, population levels remained stable until the end of the assay with a population of 1.50 $\log_{10}$ CFU/cm^2 (day 16). By PMA-qPCR, the population levels started at 4.16 $\log_{10}$ cells/cm^2 and decreased progressively until 2.21 $\log_{10}$ cells/cm^2 at day 7. After 16 days, the population quantification of CPA-2 was not quantified, due to no amplification was obtained in sampling. It is important to point out that the lower limit of cells detectable by qPCR is 2.17 $\log_{10}$ cells/cm^2 fruit surface. Finally, in the quantification of CPA-2 by qPCR, the initial population levels were approximately 4.60 $\log_{10}$ cells/cm^2, at day 7 decreased until reaching a population of 3.00 $\log_{10}$ cells/cm^2 and remained stable until the end of the assay (16 days).

Figure 2B shows the population levels of CPA-2 recovered from the surfaces of INS-fruits of treated trees and in theory less exposed fruits to the treatment. The *P. agglomerans* population quantified by dilution plating was approximately 4.25 $\log_{10}$ CFU/cm^2 at time 0 and decreased during first 3 days until 3.25 $\log_{10}$ CFU/cm^2. Then, population levels decreased drastically at day 7 (1.45 $\log_{10}$ CFU/cm^2) and then remained constant until the end of the assay. By PMA-qPCR, the population levels started at 4.10 $\log_{10}$ cells/cm^2 and decreased progressively until 2.68 $\log_{10}$ cells/cm^2 at day 7. At day 16, the population quantification was not possible, due because cell numbers were below quantification limit of qPCR. Finally the quantification of CPA-2 by qPCR, started at approximately 4.70 $\log_{10}$ cells/cm^2, slightly decreased and remained stable during the first 16 days after treatment (approximately 3.90 $\log_{10}$ cells/cm^2).

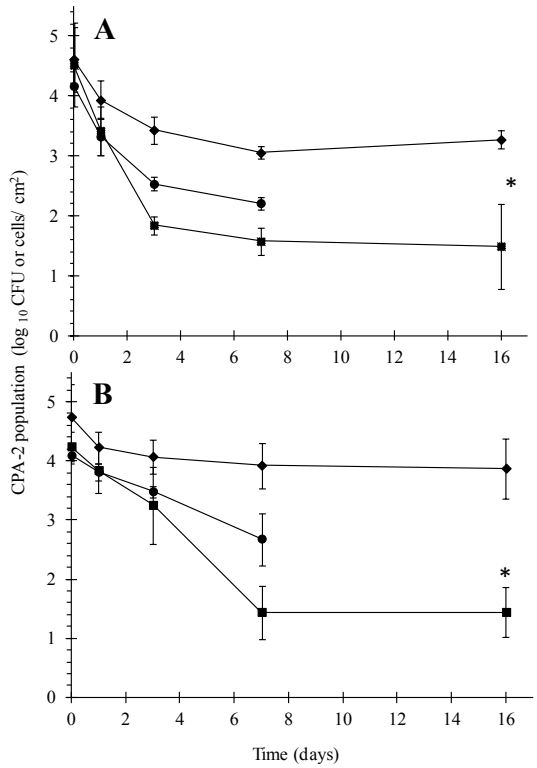

Figure 2. Population dynamics of *P. agglomerans* CPA-2 estimated by PMA-qPCR (●), qPCR (♦) and plating (■) on the orange surface. CPA-2 treatment was sprayed on Valencia cultivar orange trees two weeks before harvest. Sample fruits were collected from (A) the sun-exposed (designated as "OUT-fruits") and (B) shaded canopy position (designated as "INS-fruits"). (*) Under threshold. The points represented the means of four biological replicates, and the vertical bars indicate the standard deviation of the mean.

4.3.3. Evaluation of spray dispersion of *P. agglomerans* CPA-2 treatment

Spray dispersion of *P. agglomerans* treatment after its application on citrus trees is shown in Figure 3. Results show significant differences in the percentage coverage between treated and non-treated trees (Figure 3A). Mean spray cover was smaller in non-treated trees than the treated trees. Mean values of the percentage spray cover on WSP placed on treated trees were 63 % on outer-upward and 20 % on outer-downward facing papers. Further, the percentage spray cover was 45 % and 4 % on inner-upward and inner-downward facing papers, respectively. The percentage spray cover on WSP placed on non-treated trees was less than 1 %, both outer and inner canopy of the tree. No statistically significant differences were observed comparing the percentage spray cover of WSP placed outer and inner of non-treated trees. The values obtained were

0.4 % and 0.2 % on inner-upward and inner-downward facing papers, respectively. Then, the coverage percentage was almost negligible in the non-treated trees.

Figure 3B showed the relationship between percentage spray cover and distance between treated and non-treated trees. Consistently, the greatest spray cover was observed in samples under treated trees (ie, distances smaller than 0.5 m).The coverage percentages varied between 20 and 30 % in distances greater than 0.5 m, but less than 1.5 m. Distances more than 2.0 m showed percentages of spray cover values below to 5 %. Finally, in water sensitive papers placed under non-treated trees (ie, distances of 2.5 to 3.5 m) drops were not observed, which could indicate lack of spray dispersion.

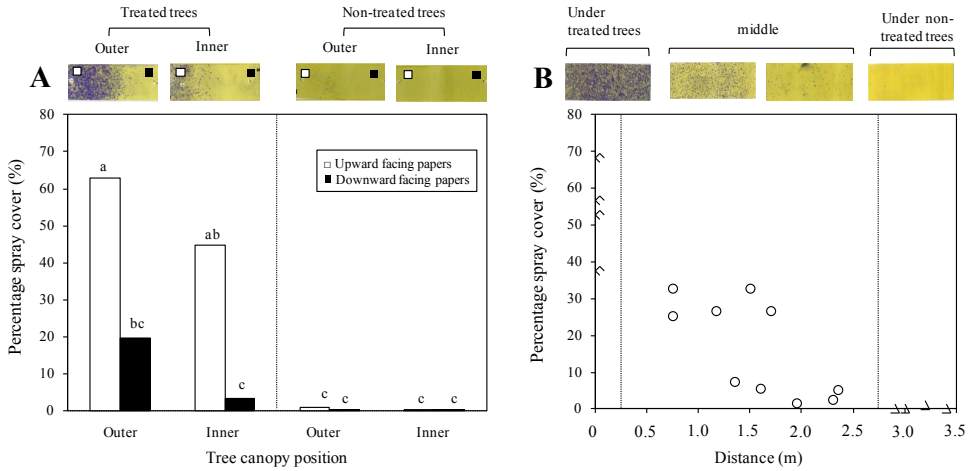

Figure 3. Percentage of the surface area of water-sensitive papers covered with spray of *P. agglomerans* treatment. CPA-2 treatment was sprayed on Valencia cultivar orange trees two weeks before harvest. The water sensitive papers (WSP) were placed: (A) on "outer" (empty square) and "inner" (full square) canopy of the tree both treated and non-treated trees, and (B) on soil around of treated (◊) and non-treated (Δ) trees, and between trees at 1/2 or 2/3 tree of distance between both treated and non-treated trees (O). Means with the same letter for each situation are not significantly different ($P < 0.05$) according to Student's *t*-test.

4.3.4. Study of environmental distribution and persistence of *P. agglomerans* CPA-2 in preharvest application

Population dynamics of *P. agglomerans* CPA-2 isolated from treated area is shown in Figure 3. The population of *P. agglomerans* on weed determined by dilution plating started at 6.11 $\log_{10}$ CFU/g decreased slightly during the first 3 days and remained stable, with a population of approximately 4.96 $\log_{10}$ CFU/g at day 21 (Figure 4A). The

estimated population of CPA-2 strain identified by conventional PCR was approximately 5.00 $\log_{10}$ CFU/g immediately after treatment (day 0). During the first 7 days after treatment, the CPA-2 populations decreased progressively until 2.10 $\log_{10}$ CFU/g and then remained stable until 16 days. After 16 days, the CPA-2 population decreased considerably until reached 0.31 $\log_{10}$ CFU/g at the last sampling (21 days). The population with phenotypic characteristics similar to *P. agglomerans* monitored by plating was approximately 94 % higher than the percentage of CPA-2 identified by PCR at day 21 after the treatment application. Some of these colonies with phenotypic characteristics similar to *P. agglomerans* not identified as CPA-2 by PCR were identified by sequencing 16s ribosomal DNA, as *Pantoea* spp. and *Pantoea ananatis*. Moreover, from ground sampling; CPA-2 was not detectable at any time after application with systems, dilution plating and PCR (data not shown).

Figure 4B shows the population levels of CPA-2 recovered from OUT-leaves and INS-leaves of treated trees. Initial populations of *P. agglomerans* were 5.56 and 5.76 $\log_{10}$ CFU/cm^2 on both OUT-leaves and INS-leaves, respectively. At day 1, population levels observed outside leaves with respect to populations recovered from INS-leaves were significantly different; 3.96 $\log_{10}$ CFU/cm^2 and 4.81 $\log_{10}$ CFU/cm^2, respectively. Population decreased during the next two days and then remained stable until day 7. No differences between OUT-leaves and INS-leaves were observed. After this time, the population levels of CPA-2 decreased progressively until sampling day 16. At the end of the assay (16 days), the population recovered from OUT-leaves was 1.57 and 1.96 $\log_{10}$ CFU/cm^2 from INS-leaves. No statistical differences were observed between the population quantified by dilution plating and the one identified by PCR. Results of sampling conducted on non-treated area are shown in Table 1. At sampling day 0, all petri dishes from air sampling showed presence of colonies with phenotypic characteristics similar to *P. agglomerans*, and all colonies were identified as CPA-2 by conventional PCR. The colonies similar than *P. agglomerans* from 1 to 16 days decreased progressively from 69 to 13 %, respectively. And the CPA-2 colonies confirmed by PCR were always less than 9 %. At sampling day 21, CPA-2 strain was not detected in the air.

In weeds, the CPA-2 strain was detected and molecularly confirmed at both 0 and 1 day on 100 % of plates. At sampling day 3, colonies with phenotypic characteristics similar to *P. agglomerans* were detected in all the Rodac plates, but only 38 % of colonies were confirmed by PCR as CPA-2. And at sampling day 7 and 16, the presence of CPA-2 strain was confirmed in 50 and 13 % of Rodac plates, respectively. At day 21, 38 % of samples presented colonies with phenotypic characteristics similar to *P. agglomerans*, but neither of them were identified as CPA-2.

In leaves, CPA-2 strain was detected and confirmed at both 0 and 1 day but levels lower than 10 %. At sampling day 3, CPA-2 was not detected; however, at sampling day 7, 38 % of samples showed colonies with phenotypic characteristics similar to *P. agglomerans*, and 25 % of colonies were confirmed as CPA-2 by conventional PCR. At sampling day 16 and 21, CPA-2 was not confirmed in any plate. Consequently, the persistence of CPA-2 is less than 16 days in leaves of non-treated trees.

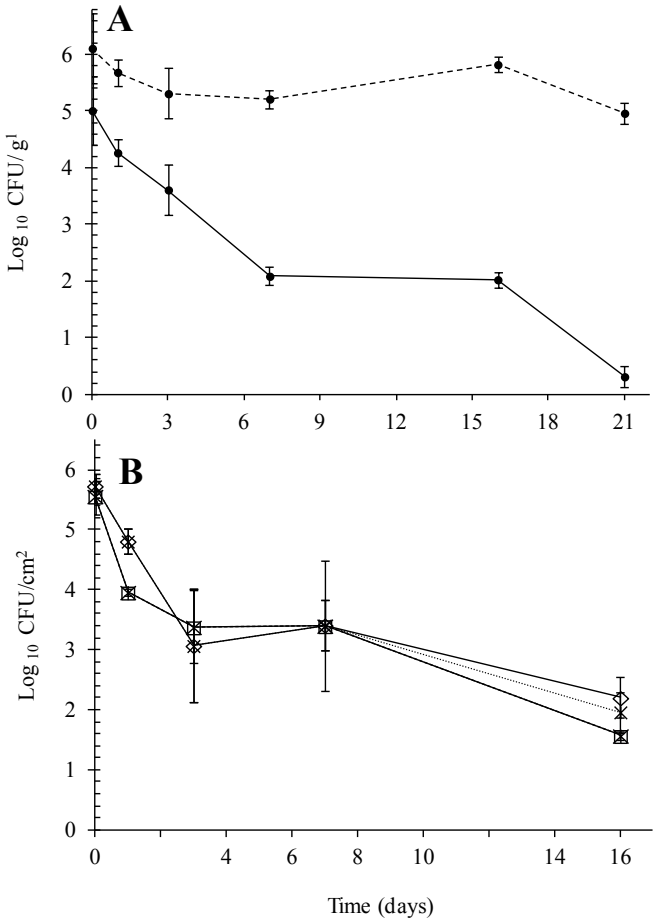

Figure 4. Population dynamics of *P. agglomerans* CPA-2 isolated from treatment area. The sampled points were: (A) weed (●), and (B) OUT-leaves (□,x) and INS-leaves (◇,x). CPA-2 treatment was sprayed on Valencia cultivar orange trees two weeks before harvest. Population was monitored by dilution plating (discontinued line) and confirmed by conventional PCR (continued line). The points represented the means of four biological replicates, and the vertical bars indicate the standard deviation of the mean.

On fruit surface just after treatment, only 50 % of colonies with phenotypic characteristics similar to *P. agglomerans* were confirmed as CPA-2. At sampling day 1 and 3 any colony observed with phenotypic characteristic in Rodac plates was identified as CPA-2 by conventional CPA-2. However, at sampling day 7, on 50 % of samples showed colonies with phenotypic characteristics similar to *P. agglomerans*, but only 13 % was confirmed as CPA-2 by PCR. The persistence of CPA-2 on fruits was less than 16 days, because the CPA-2 strain was not confirmed by PCR after this time.

Table 1. Total sampling plates from non-treated area that showed presence of colonies with phenotypic characteristics similar to *P. agglomerans* that were identified by conventional PCR to be CPA-2.

Sample	Sampling time (days)	Phenotypically similar to CPA-2 [a] (%)		CPA-2 confirmed by PCR[b] (%)	
Air	0	32/32	(100)	32/32	(100)
(*n*=32)	1	22/32	(69)	3/32	(9)
	3	12/32	(38)	1/32	(3)
	7	9/32	(28)	3/32	(9)
	16	4/32	(13)	2/32	(6)
	21	0/32	(0)	-	-
Weeds	0	8/8	(100)	8/8	(100)
(*n*=8)	1	8/8	(100)	8/8	(100)
	3	8/8	(100)	3/8	(38)
	7	6/8	(75)	4/8	(50)
	16	5/8	(63)	1/8	(13)
	21	3/8	(38)	0/8	(0)
Leaves	0	5/8	(63)	3/8	(38)
(*n*=8)	1	3/8	(38)	1/8	(13)
	3	1/8	(13)	0/8	(0)
	7	3/8	(38)	2/8	(25)
	16	1/8	(13)	0/8	(0)
	21	0/8	(0)	-	-
Fruits	0	6/8	(75)	4/8	(50)
(*n*=8)	1	1/8	(13)	0/8	(0)
	3	2/8	(25)	0/8	(0)
	7	4/8	(50)	1/8	(13)
	16	1/8	(13)	0/8	(0)
	21	0/8	(0)	-	-

[a] Number of plates that showed presence of colonies with phenotypic characteristics similar to *P. agglomerans* in relation to the total number of plates.

[b] Number of plates that showed presence of CPA-2 colonies confirmed by conventional PCR in relation to the number of plates that showed the presence of colonies with phenotypic characteristics similar to *P. agglomerans*.

Finally, the results of sampling conducted on the motorized backpack sprayer, gloves and working clothes worn by handler are shown in Table 2. The sample from the motorized backpack sprayer before treatment did not show the presence of strain CPA-2. Immediately after treatment, CPA-2 was detected in all samples. Washing motorized backpack sprayer only with water was not sufficient to completely remove CPA-2.

Table 2. Total sampling plates from motorized backpack sprayer and handlers clothes that showed presence of colonies with phenotypic characteristics similar to *P. agglomerans* that were identified by conventional PCR to be CPA-2.

Sample	Sampling time (days)	Phenotypically similar to CPA-2 [a] (%)		CPA-2 confirmed by PCR[b] (%)	
Motorized backpack sprayer	Before treatment	0/5	(0)	-	
	After treatment	5/5	(100)	5/5	(100)
(*n*=5)	After washing	5/5	(100)	5/5	(100)
	1	4/5	(80)	4/5	(80)
	2	3/5	(60)	3/5	(60)
	5	2/5	(40)	1/5	(20)
	7	0/5	(0)	-	-
Working clothes	0	9/9	(100)	9/9	(100)
(*n*=9)	1	8/9	(89)	7/9	(87.5)
	3	3/9	(33)	2/9	(66.7)
	5	2/9	(22)	2/9	(100)
	7	0/9	(0)	-	-
Gloves	0	4/4	(100)	4/4	(100)
(*n*=4)	1	1/4	(25)	1/4	(25)
	2	0/4	(0)	-	-

[a] Number of plates that showed presence of colonies with phenotypic characteristics similar to *P. agglomerans* in relation to the total number of plates.

[b] Number of plates that showed presence of CPA-2 colonies confirmed by conventional PCR in relation to the number of plates that showed the presence of colonies with phenotypic characteristics similar to *P. agglomerans*.

Indeed, CPA-2 strain was still confirmed after the sampling at day 5. However, at sampling day 7, CPA-2 was not detected on motorized backpack sprayer. On the working clothes, the CPA-2 was isolated and confirmed at day 0 in all samples, during first the 5

days; the persistence of CPA-2 decreased progressively and completely disappeared after 7 days. In addition; on the gloves worn by handlers at sampling day 0, CPA-2 was detected in 100 % samples. At sampling day 1, only 25 % of samples showed colonies of *P. agglomerans* CPA-2. The persistence of the BCA on the gloves was less than 2 days, because the CPA-2 strain was not detected after this time.

4.4. DISCUSSION

Monitoring the environmental presence of the BCA *P. agglomerans* CPA-2 is important for understanding its interaction(s) with its environment and for ecological and safety assessments. Dilution plating is the quantification method most commonly used to monitor BCAs (Teixidó *et al.* 1998; Cañamás *et al.* 2008; De Cal *et al.* 2009). This method has the advantage that only viable microorganisms are detected, but they would have a lack of specificity if there were non-targeted microorganisms in the environment (Schena *et al.* 2013). Methods based on acid nucleic, as qPCR, have become more widespread for being extremely sensitive and highly specific (Sanzani *et al.* 2014). Quantitative PCR method has been used for quantitative detection of BCAs, in mould (Larena and Melgarejo, 2009), yeasts (Schena *et al.* 2002; Massart *et al.* 2005; Spotts *et al.* 2009) and bacteria (Pujol *et al.* 2006; Braun-Kiewnick *et al.* 2012) used to control postharvest decays. It is assumed that a major disadvantage of this method was its inability to differentiate between viable and nonviable cells and the overestimation of potentially viable population (Soto-Muñoz *et al.* 2014). In recent years, DNA-intercalating dye PMA has been used successfully together with qPCR reaction to discriminate and count both live and dead cells in microbiological samples (Nocker and Camper, 2009; van Frankenhuyzen *et al.* 2011).

In the present work, we have monitoring the population of biocontrol agent *P. agglomerans* CPA-2 on citrus fruit treated in preharvest by three methods: dilution plating, PMA-qPCR and qPCR. Our results showed the highest population levels were determined by qPCR, followed by PMA-qPCR and dilution plating. The initial population of CPA-2 quantified by dilution plating was around 4.5 $\log_{10}$ CFU cm^{-2} (day 0). These results are consistent with those reported in a study conducted by Cañamás *et al.* (2008a), who determined population of lyophilized *P. agglomerans* CPA-2 cells osmotically adapted, applied on orange citrus during springtime environmental conditions. In contrast, the population quantified in this study by dilution plating during the first 3 days after treatment application was lower that population found by Cañamás *et al.* (2008), at 4 days after treatment application. It could be due to the effect that field conditions during the present experiment (relative humidity and temperatures) were harsh than the environmental conditions registered during experiment performed

before. In general, the cultivable population of CPA-2 showed more stability on INS-fruits than OUT-fruits along the sampling. Probably, OUT-fruits are more exposed to factors such as light intensity, humidity, wind, irradiation and high temperature than INS-fruits.

To our knowledge, this is the first study that has used the PMA-qPCR as monitoring method of BCAs applied on fruit under field conditions. Population levels quantified by PMA-qPCR were significantly different than those obtained by dilution plating method after 3 and 7 days of field treatment INS-fruits and OUT-fruits, respectively. This difference might be attributed to that PMA dye can detect live cells, as cells metabolically active in a VBNC state. In fact, VBNC state is a survival strategy of many bacteria in the environment in response to adverse environmental conditions (Oliver, 2010). Furthermore, PMA could detect cells that have lost their metabolic activity, but their membrane is still kept intact, resulting in their inability to grow on a plate medium (Nocker and Camper, 2009). An evident difference between PMA-qPCR and qPCR accounts was observed; this difference suggests the ability of PMA to selectively inhibit amplification of DNA derived from non-viable cells amplification during qPCR. In food microbiology, several studies have proven the usefulness of PMA-qPCR to discriminate and quantify the live and dead cells present in different types of bacterial samples, using pure culture, environmental and food samples (Elizaquível *et al.* 2014).

Population levels of CPA-2 quantified by qPCR were not statically significant compared with populations quantified by PMA-qPCR and dilution plating, only during the first day after application. However, qPCR showed an overestimation of the population of CPA-2 compared with PMA-qPCR and dilution plating method during longer times after application (3 to 16 days). This finding has been also found in other studies conducted with BCAs applied at field and postharvest conditions (Pujol *et al.* 2006; Soto-Muñoz *et al.* 2014). In both studies, these authors attributed the overestimation at long periods of time to the stressful conditions of the environment that can promote that cells entry into a VBNC state and the presence of free DNA after cell death. It is known that DNA degradation rate strongly depends on environmental conditions (Schena *et al.* 2004). Some research has demonstrated that DNA free was rapidly degraded in ground (Schena and Ippolito, 2003). In contrast, other studies have demonstrated the persistence of DNA during a long period of time after cell death in soil (England *et al.* 1997). Our results showed that DNA of *P. agglomerans* CPA-2 can persist after cell death in a detectable form by qPCR for at least 16 days in both OUT-fruits and INS-fruits.

The spray dispersion of CPA-2 treatment in the environment was evaluated. The maximal values of percentage spray cover were observed outside of treated trees and negligible amounts of spray treatment (less 1 %) were detected outside of non-treated

trees (Figure. 2A). Moreover, the results showed a consistent inverse relationship between spray cover and tree distances. Under our experimental conditions, lack dispersion of spray treatment was observed at distances of 2.5±0.5 m. However, Some caution needs to be exercised in interpreting these results due to during spray application can be generated finer sprays, undetectable in the water sensitive papers, and it produce micro-distributions of deposits on the plant surface (Cross *et al.* 2001).

The presence and persistence of CPA-2 in the environment were monitored in different areas by conventional dilution plating method and confirmation of cultured colonies by conventional PCR, using primers PAGA1 and PAGB1. In the treatment area, the results showed that CPA-2 can survive but not proliferate in weed and leaves. Surprisingly, just an important number of colonies with morphology similar than *P. agglomerans* were observed in weed, but neither of them corresponded to CPA-2 strain. This fact could explain the significant difference found between populations quantified by dilution plating and percentage of colonies identified as CPA-2 by conventional PCR. The *Pantoea* species are widely distributed in nature and has been isolated from numerous ecological niches, including weed (Gitaitis *et al.* 2002). Microbial weed species are commonly related with species, such as *Acinetobacter johnsonii*, *Alkanivorax borkumensis*, *Pseudomonas putida* and *Pantoea ananatis* (Cray *et al.* 2013). *P. ananatis* and *P. agglomerans* are closely related, at one time they were considered to be the same species (Hauben *et al.* 1998). These species to the genus *Pantoea* are difficult to identify, owing to high phenotypic similarity, a lack of distinguishing characteristics and a somewhat confusing taxonomy (Deletoile *et al.* 2009). In our study was not possible to differentiate CPA-2 to other for colony morphology in weed samples. In contrast, the use of SCARs primers was a useful tool to identify the antagonist and distinguish from other strains of the same species in the environment. We also found that CPA-2 cannot survive in the ground. The physical properties of substrates are critical determinants for the soil persistence of a BCA following its application (Vänninen *et al.* 2000). Vázquez *et al.* (2013) also reported that the persistence of the BCA, *Penicillium oxalicum* strain 212 in soil substrates was limited.

Monitoring of CPA-2 in non-treated area showed that persistence of CPA-2 was lower than 21 days in the air and weed. CPA-2 persistence on leaves and fruit surface was lower than 16 days. The persistence of the BCA on inert surfaces, such as, motorized backpack sprayer and working clothes was lower than 7 days, and lower than 2 days in gloves. According our results, CPA-2 strain has a low persistence and it had not indication of permanent establishment in the citrus field. The persistence of CPA-2 may be influenced by the tolerance of antagonist to the environmental stresses such as dry conditions, direct UV irradiation, high temperatures, low nutrient availability, and rapid climatic changes (Nunes, 2012). Any of these stress factors may result in sublethal injury to a portion of

the population (Kinkel *et al.* 2000). Although, in application in foliar environments (which were our case), solar radiation and specially UV portion of the spectrum, is probably the most important factor affecting the persistence of a BCA (Filho *et al.* 2001). Thus, our results demonstrate that *P. agglomerans* CPA-2 would not present an unacceptable risk for the environment due to its low establishment, survival and dispersal in the environment.

4.5. CONCLUSIONS

In general, these advances provide a better understanding of the environmental fate and persistence of field-applied antagonist and will lead to an optimization of formulations, time and mode of application, with beneficial effects on the level of protection that can be achieved, without disregarding the possible risk for the environment. Moreover, these advances provided new possibilities for insight into ecophysiological constraints and may be used to generate valuable data for registration purposes.

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CAPÍTULO V

Molecular tools applied to identify and quantify the biocontrol agent *Pantoea agglomerans* CPA-2 in postharvest treatments on oranges

Soto-Muñoz, L. Teixidó, N. Usall, J. Viñas, I. Abadias. M. Torres, R.

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ABSTRACT

Pantoea agglomerans strain CPA-2 is an effective biocontrol agent (BCA) for postharvest diseases of citrus and pome fruits. To implement their use as control strategy is necessary to study the traceability of BCA in environment application for registration issues. In this study, the presence and persistence of CPA-2 were monitored in packing line, storage chambers and working clothes by PCR conventional. After postharvest application, the presence of CPA-2 was not detectable in the environment and storage chambers, whereas on working clothes and packing line its persistence was less than 1 and 3 days, respectively. Additionally, CPA-2 population was quantified on oranges stored at two different temperatures (20 and 4 °C) by quantitative PCR (qPCR), sample pretreatment with a propidium monizade dye (PMA-qPCR) and dilution plating method. At the initial time of the assay, no differences were observed in CPA-2 population quantified by qPCR, PMA-qPCR and dilution plating, at both storage temperatures. However, CPA-2 populations quantified by PMA-qPCR were significantly different compared with that obtained by qPCR method along time-course of the assay; not significant differences were observed between PMA-qPCR and dilution plating. In conclusion, the persistence of *P. agglomerans* CPA-2 on different sampling areas after postharvest application was low. Furthermore, PMA-qPCR method gave valuable information on viable population behavior and the presence of residual DNA from dead cells. In general, these studies help to understand the persistence of antagonist when it is applied under postharvest conditions and will lead to an optimization of time and mode of application

Keywords: Orange, molecular marker, qPCR, persistence, viable cells, PMA.

5.1. INTRODUCTION

Postharvest green mould, caused by *Penicllium digitatum* (Per:Fr) Sacc, and postharvest blue mould, caused by *Penicillium italicum* Wehmer, are among the most economically important postharvest diseases of citrus worldwide (Talabi *et al.* 2014). Although the use of synthetic chemical fungicides remains a primary method of controlling postharvest diseases, the global trend appears to be shifted towards reduced the use of fungicides on produce and hence there is a strong public and scientific desire to seek safer and eco-friendly alternatives to reduce postharvest losses (Mari *et al.* 2007). Biological control using microbial antagonists such as bacteria and yeasts has emerged as one of the most promising of these alternatives (Droby *et al.* 2009). The potential antagonist activity of many biocontrol agents (BCAs) for controlling postharvest diseases of fruit has been clearly demonstrated (Teixidó *et al.* 2011). In this context, the BCA *Pantoea agglomerans* strain CPA-2 has been demonstrated to be effective against the main postharvest diseases affecting citrus fruits (Teixidó *et al.* 2001; Plaza *et al.* 2004; Torres *et al.* 2007; Usall *et al.* 2008). At present, the formulated product, is now available although it is not being commercialized (Torres *et al.* 2014).

The main goal of the development and implementation of a biocontrol product is to improve the ability of the BCA to successfully control postharvest diseases under a wider array conditions and with minimal variability (Droby *et al.* 2003). And an effective decay control depends on the ability of antagonist to colonize the surface of fruit in both field and storage conditions and also to persist for as long as possible is vitally important (Wisniewski and Wilson, 1992). In addition, for its commercialization the registration of a biocontrol product is required before any commercial use, as a usual food safety product. For EU member states, the specific requirements for registering a BCA can be found in Regulation 1107/2009, which updates the existing regulations and replaces Directive 91/414/EEC. Some requirements of the directive concerns fate and behavior of the microorganism in the environment and its impact on non-target species and the residues. To address the fate of an introduced microorganism in the environment the most novel approach consists in designing a SCAR marker (specific-characterized-amplified-region) that will enable the natural organism to be traced among other strains of the same species in the environment (Alabouvette and

Cordier, 2011). Strain-specific SCAR markers have been developed for some BCAs applied in postharvest (Schena *et al.* 2002; De Clercq *et al.* 2003; El Hamouchi *et al.* 2008) including *Pantoea agglomerans* strain CPA-2 (Nunes *et al.* 2008).

Although conventional PCR using SCAR markers has become an attractive tool for the detection of specific microorganisms in microbial systems, this technique does not allow accurate quantification of DNA. This shortcoming has been overcome by the emergence of new techniques that can quantify nucleic acids in vitro. Real-time quantitative PCR (qPCR) is one of these techniques. qPCR is a sensitive and automated high-throughput technique that allows specific detection and quantification of BCAs (Larena *et al.* 2005; Massart *et al.* 2005; Pujol *et al.* 2006; Spotts *et al.* 2009; Edel-Hermann *et al.* 2011; Braun-Kiewnick *et al.* 2012; Soto-Muñoz *et al.* 2014b). In addition, recently it has been demonstrated that qPCR can be used to distinguish between DNA from dead and live cells by including a pretreatment of the sample with a DNA intercalating reagent, e.g. propidium monoazide (PMA) (Nocker *et al.* 2006). This procedure is based on the integrity of bacterial cells since PMA penetrates only into compromised membrane cells (Nocker and Camper, 2009). Sample pretreatment with PMA combined with qPCR (PMA-qPCR) has been successfully tested for selective detection and quantification of foodborne pathogens (Chen *et al.* 2011; Elizaquível *et al.* 2012; Dinu and Bach, 2013) and other relevant microorganisms in the food industry (Andorra *et al.* 2010; Crespo-Sempere *et al.* 2013), but not for BCAs. In previous work, the ideal conditions for differentiate CPA-2 viable in cell suspensions and on a matrix of orange peel have been assayed (Soto-Muñoz *et al.* 2014a).

In this work, we used qPCR combined with PMA to quantify the populations of *P. agglomerans* CPA-2 applied on oranges in postharvest conditions. In addition, we compared these results with that obtained by dilution plating and qPCR. We also monitored the persistence and dispersion of CPA-2 on different environments as packing line, storage chambers and working clothes.

5.2. MATERIAL AND METHODS

5.2.1. Fruit

Oranges cv *Valencia Late* were used in all experiments. Fruits were obtained immediately after harvests from commercial orchard located in the Montsià area, South of Tarragona (Catalonia, Spain). Fruits were selected by hand for uniformity of size from trees without any treatment after harvest. The fruits were stored without any chemical postharvest treatment at 4 °C and 85 % relative humidity (RH) before use.

5.2.2. Antagonist

The CPA-2 strain of *P. agglomerans* used in this study was obtained from IRTA Centre in Lleida (Catalonia, Spain). This strain was isolated from Golden Delicious apples and is currently deposited at the Spanish Collection of Type Culture (CECT, University of Valencia, Valencia, Spain), as CECT-4920. Stock cultures were stored long-term at -80 °C in cryogenic vials and subcultured on nutrient yeast dextrose agar (NYDA: 8 g/L nutrient broth, 5 g/L yeast extract, 10 g/L dextrose and 20 g/L agar). The activated culture was maintained on NYDA at 30 °C for 24 h and transferred to potassium phosphate buffer (pH 6.5) (0.2 M KH_2PO_4, 70 mL; 0.2 M K_2HPO_4, 30 mL and deionised water, 300 mL) to obtain a cell suspension. This suspension was used as an inoculum for biomass production in a fermentation system. Osmotically adapted cells grown in liquid medium (5 g/L yeast extract, 10 g/L sucrose and 25 g/L NaCl), as previously described Cañamás *et al.* (2008b), were used in all studies. An appropriate volume of inoculum was added to 5 L of the liquid medium adjusted to 1 x 10^6 CFU/mL. Cultures were grown in a 5 L bench-top BIOSTAT-A fermenter (Braun Biotech International, Melsungen, Germany) at 30 °C with 300 rpm agitation and 100 L/h aeration. Cultures were harvested at stationary phase (24 h) by centrifugation at 9820 *g* for 10 min at 15 °C in an Avanti™ J-25 centrifuge (Beckman, Palo Alto, Ca, USA). Cell pellets were resuspended in deionised water containing 10 % sucrose, frozen at -20 °C overnight and freeze-dried (Cryodos, Telstar SA, Terrasa, Catalonia, Spain) at 1 Pa and -45 °C for 24 h as described by Costa *et al.* (2000). These lyophilised cells were packaged in a white high-density polyethylene bottle at non-vacuum atmosphere (Torres *et al.* 2014). For the postharvest application of biocontrol agent, cell pellets were resuspended in 1 % non-fat skim milk (Sveltesse, Nestle, Vevey, Switzeerland) and used immediately to treat oranges.

5.2.3. Semi-commercial trial using *P. agglomerans* CPA-2

A semi-commercial trial using a packing line was conducted at IRTA-Centre in Lleida. Fruits were sprayed with the formulated and packed product of *P. agglomerans* CPA-2 previously described and adjusted to a concentration of 2 x 10^9 CFU/mL following standard industrial procedures in the packing line. Untreated fruits were used as control. After treatment, oranges were placed in ten plastic boxes containing 60 fruit each. Fruit boxes were separated into two sets: one set was stored at 20 °C and 80 % RH for 9 days, and the other set at 4 °C and 85 % RH for 30 days.

5.2.4. Quantification of *P. agglomerans* CPA-2 on orange surface

The quantification of *P. agglomerans* CPA-2 on orange stored at 20 °C and 4 °C was carried out by different quantification methods. The quantification methods were: *(i)*

dilution plating to obtain the active-culturable population *(ii)* PMA-qPCR technique to obtain viable cell counts, and *(iii)* qPCR to obtain the total cell count. The recovery of CPA-2 cells from orange surfaces was performed according to the method described by Torres *et al.* (2012), with minor modifications. Briefly, eight pieces of peel (16 mm diameter) were removed from each fruit. Peel samples were mixed with 20 mL of 0.05 M phosphate buffer solution in a sterile stomacher filter bag and pummelled in a Stomacher 400 set at normal speed for 90 s resulting mixture was filtered through a 11 µm porous filter (Whatman® International, Maidstone England). Five fruits constituted a single replicate, and four biological replicates were performed. For quantification by dilution plating, ten-fold dilutions were made in phosphate buffer solution and plated on NYDA media. Each suspension was analyzed twice. The total number of CFU was counted after incubation of plates at 30 °C for 24 h. For analysis by PMA-qPCR and qPCR, 8 mL of each suspension sample were split in two equivalents samples. One of them was used for PMA treatment as described above and the other one was used for DNA extraction as described in Section 5.2.4.2. The amount of resulting CPA-2 cells was calculated according to the generated standard curve by qPCR described in Section 5.2.4.2.

5.2.4.1. PMA treatment

Treatment of samples with PMA (phenanthridium, 3-amino-8-azido-5-[3- (diethylmethylammonio)propyl]-6-phenyl dichloride; Biotum, Hayward, USA) was performed based on optimization previously described by Soto-Muñoz *et al.* (2014a). Briefly, 4 mL of the sample suspension was treated with 30 µM PMA, 20 min of dark incubation and 30 min of LED light exposure. Afterwards photo-induced cross-linking, cells were centrifuged at 9727 *g* for 20 min in a Hettich Mikro 22R centrifuge (Hettich Zentrifugen, Tuttlingen, Germany) prior to DNA extraction, followed by qPCR.

5.2.4.2. DNA extraction and qPCR

Genomic DNA of *P. agglomerans* CPA-2 was extracted using Lyse-N-Go PCR Reagent (Thermo Scientific, Rockford, IL, USA) following the manufacturer's instructions. The amount and purity of DNA samples were determined spectrophotometrically (NanoDrop ND-1000; NanoDrop Technologies, Wilmington, DE) and DNA integrity was evaluated by electrophoresis on 1.5 % agarose gel run at 70 V for 90 min with TEB buffer and stained with 0.5 mg/µL ethidium bromide. The ABI-7500 qPCR detection system (Applied Biosystem Division, Perkin-Elmer Co., Foster City, CA, USA) was used to quantify genomic CPA-2 DNA. PCR reactions were performed in a final volume of 25 µL containing 1× PCR *Taqman* Universal PCR Master Mix (Applied Biosystem, USA), 300 nM of SP_2-forward primer (CTTAAAGCGCAGGGAAGCCGGTCAG), 300 nM of SP_2-reverse primer (GAGCCGGCTCAGGGAAACCGGTC), 200 nM of *Taqman* MGB

probe (FAM-TCCATGGATGGCTTAAG-MGB) defined in a previous work (Soto-Muñoz *et al.* 2014b) and 4 µL of DNA extracted with and without use of PMA treatment diluted ten-fold. Cycling conditions used were: 2 min at 50 °C, 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 59 °C. Two negative controls were included: *(i)* using DNA extracted from untreated fruits and *(ii)* using water instead of genomic DNA. Data acquisition and analysis were performed using SDS software version 1.3.1 (Applied Biosystems) according to the manufacturer's instructions. The quantification cycles (*Cq*) values were automatically calculated for each reaction by the SDS software. Any sample that reached a fluorescence value exceeding the fluorescence threshold value was considered positive.

A standard quantification curve was prepared by adding a range of bacterial cell concentrations (from 1.5×10^2 to 1.5×10^6 cells/cm^2) to orange suspensions. These orange suspensions were obtained from 40 pieces of peel (16 mm diameter) of five untreated fruits in 20 mL of 0.05 M phosphate buffer solution, pummelled in a Stomacher 400 set at normal speed for 90 s. DNA extraction was done as described above. The *Cq* values obtained were used to plot a standard curve by assigning the corresponding concentration values. Standard curve obtained was $y = -3.08x + 049.68$; $R^2 = 0.99$.

5.2.5. Study of dispersion and persistence of *P. agglomerans* CPA-2 in postharvest applications

Three sample types were selected to monitor CPA-2 on different surfaces and environments: *(i)* packing line, *(ii)* storage chambers (at 20 °C and 4 °C) and *(iii)* gloves and working clothes worn by handlers. All surfaces were sampled with Rodac (replicate organism direct agar contact) plates containing NYDA medium with 0.5 g/L imazalil by contact between the culture medium and the surface, with a slight pressure to allow adhered microorganisms to reach the medium. The air of each zone was also sampled according to the gravimetric method. Petri dishes containing NYDA medium supplemented with imazalil (imazalil sulphate 99 %, 0.5 g/L) were equidistantly distributed through each zone and left open for 2 min.

The sample points in the packing line (Figure 1) were: *(i)* pre-treatment zone constituted by surfaces of bin dumper (1) and pre-selection roller (2), *(ii)* treatment zone -this zone corresponding to area of application of CPA-2 treatment- the surfaces sampling were spray nozzles (3), plastic curtain (4) and brushes (5), and *(iii)* post-treatment zone constituted by waxing brushes (6), surfaces of drying tunnel (7), distribution belts (8), and packing table (9). Moreover, in each zone, the floor and the air were sampled. Each packing line surface was sampled before and after treatment, after washing only with water and 1, 2 and 3 days after treatment. However, the floor and the air were sampled during the assay (day 0) and 1 day after. In each zone sampling were

used two Rodac plates (replicates) for each surface of packing line and the floor, and two NYDA plates (replicates) were used for air sampling.

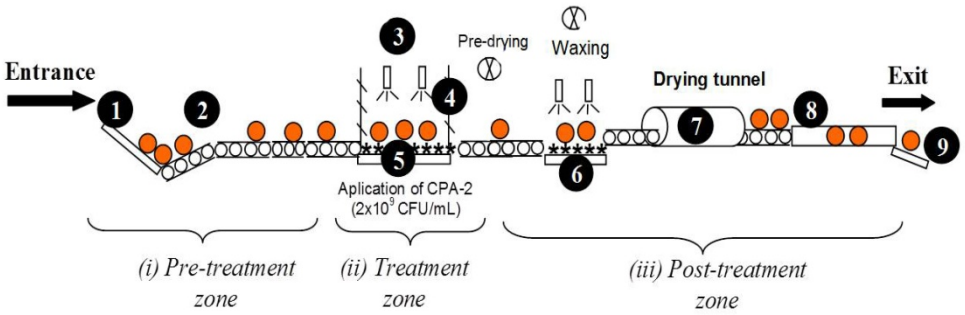

Figure 1. Sampling layout of the packing line *(i)* pre-treatment zone constituted by surfaces of bin dumper (1) and pre-selection roller (2); *(ii)* treatment zone -this zone corresponding to area of application of CPA-2 treatment- the surfaces sampling were spray nozzles (3), plastic curtain (4) and brushes (5), and *(iii)* post-treatment zone constituted by waxing brushes (6), surfaces of drying tunnel (7), distribution belts (8), and packing table (9).

In storage chambers (at 20 and 4 °C), the following locations were sampled: *(i)* the floor, walls and the air within each storage chamber just after the treatment (day 0) and 1 day after treatment and *(ii)* the surface inside and outside of two plastic boxes located in each storage chamber at days 0, 1 and 2 after treatment. For each sampling; three NYDA plates were used for air sampling, four Rodac plates were used for both the floor and on the walls in each storage chamber and two Rodac plates in each plastic box. Finally, the gloves and working clothes worn by two handlers during the semi-commercial assay were sampled at days 0 and 1 after treatment. For each sampling, two Rodac plates were used on each glove pair, and three Rodac plates were used on the working clothes of each handler.

The Rodac and NYDA plates used for the different samplings were incubated at 30 °*C* for 24-48 h. To distinguish *P. agglomerans* CPA-2 from other microorganisms, yellow colonies with phenotypic characteristics similar to *P. agglomerans* were randomly selected from plates of all sampling days. The number of selected colonies was at most 25 colonies per plate but was always at least 40 % of all colonies per plate. The selected colonies were analyzed and identified by conventional PCR test as described above.

5.2.6. Conventional PCR

PCR amplification was performed using primers PAGA1 and PAGB1 developed by Nunes *et al.* (2008). This primer pair amplifies two different fragments: a first SCAR marker (720 bp) that is specific to strain CPA-2 and the second (270 bp) specific to *P. agglomerans* species. Fifty microlitres of amplification reaction was prepared using 20 ng of each genomic DNA, 0.5 units of Taq DNA polymerase in 1×reaction buffer (Invitrogen Life Technologies, Carlsbad, CA, USA), 200 nM dNTPs and 400 nM of each SCAR primer. DNA amplification was carried out in a Peltier thermal cycler (GeneAmp PCR System 2700, Applied Biosystems) with the following programme: an initial denaturation step at 95 °C for 5 min followed by 40 cycles of 95 °C for 30 s, 59 °C for 45 s and elongation at 72 °C for 90 s followed by a final extension step at 72 °C for 10 min. The PCR products were separated by electrophoresis as described in Section 5.2.4.2. DNA standard (100 bp DNA Ladder, Invitrogen Life Technologies, Carlsbad, CA, USA) was used as a molecular size marker. The experiment was performed twice.

5.2.7. Statistical analysis

The *P. agglomerans* CPA-2 population level from three methods was calculated as CFU or cells/cm^2 of the orange surface with its standard error and a 95 % confidence interval. Population size (CFU or cells/cm^2) was log transformed to improve homogeneity of variances.

5.3. RESULTS

5.3.1. Quantification of *P. agglomerans* CPA-2 on orange surface

The quantification of *P. agglomerans* CPA-2 on oranges at two different temperatures (20 °C and 4 °C) was evaluated by three methods, dilution plating, PMA-qPCR and qPCR (Figure 2).

The *P. agglomerans* population quantified by dilution plating just after treatment and stored at 20 °C was approximately 3.70 $\log_{10}$ CFU/cm^2 (Figure 2A). Although populations decreased drastically during first day of storage (3.10 $\log_{10}$ CFU/cm^2); afterwards, they started to increase considerably reach up 3.80 $\log_{10}$ CFU/cm^2 after day 3. Then, CPA-2 populations decreased progressively until 2.60 $\log_{10}$ CFU/cm^2 at the end of the assay (9 days). By PMA-qPCR, the population levels started at 3.60 $\log_{10}$ cells/cm^2 and then decreased considerably during first day of storage to approximately 3.30 $\log_{10}$ cells/cm^2. At day 3, the CPA-2 populations increased considerably until 3.50 $\log_{10}$ cells/cm^2. Then, it decreased until 2.90 $\log_{10}$ cells/cm^2 at 9 days of storage.

Finally, the initial quantification of CPA-2 by qPCR was approximately 4.00 $\log_{10}$ cells/cm^2. After day 3, population levels increased to 4.50 $\log_{10}$ cells/cm^2 and they remained stable until the end of the assay (9 days). In general, significant differences were found between the qPCR and other two methods (PMA-qPCR and dilution plating), except at time zero. Population levels estimated by qPCR were approximately 1.20 $\log_{10}$ cells/cm^2 higher than those obtained by PMA-qPCR and dilution plating at end of the assay (9 days).

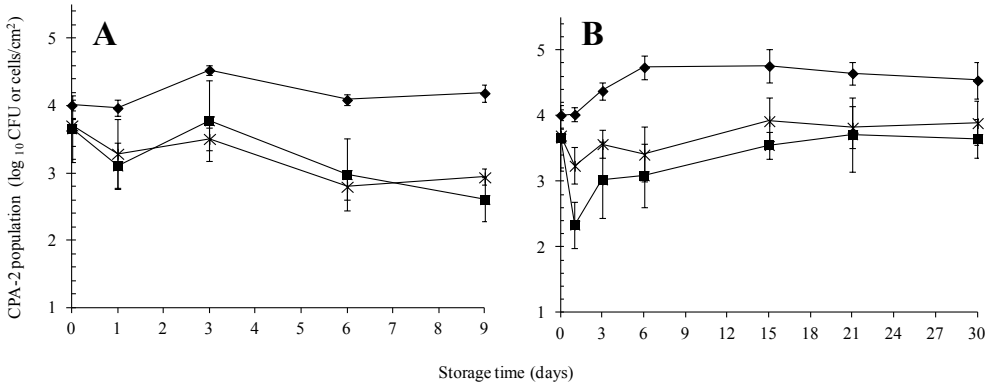

Figure 2. Population dynamics of *P. agglomerans* CPA-2 estimated by dilution plating (■), PMA-qPCR (×) and qPCR (♦) on orange surface. Fruits were treated with the bicontrol agent and stored at 20 °C (A) or under cold storage conditions at 4 °C (B). The points represented the means of four biological replicates, and the vertical bars indicate the standard deviation of the mean.

Figure 2B shows the population levels of CPA-2 recovered from the surfaces of oranges stored at 4 °C. The amount of *P. agglomerans* CPA-2 quantified by dilution plating was 3.70 $\log_{10}$ CFU/cm^2 at time 0 and decreased drastically at day 1 of storage until 2.30 $\log_{10}$ CFU/cm^2. After day 3 of storage, population levels increased considerably until 3.00 $\log_{10}$ CFU/cm^2, and continue increase up until the end of the assay (30 days) achieved 3.65 $\log_{10}$ CFU/cm^2. By PMA-qPCR, the population levels started at 3.70 $\log_{10}$ cells/cm^2 and slightly decreased to 3.24 $\log_{10}$ cells/cm^2 at day 1. At day 3, the CPA-2 population increased and they remained constant until the end of the assay. Finally the quantification of CPA-2 by qPCR, started at approximately 4.00 $\log_{10}$ cells/cm^2 and increased considerably during the first 6 days of storage until 4.70 $\log_{10}$ cells/cm^2. Then, population levels remained constant throughout of storage.

In general, populations obtained by PMA-qPCR were slightly higher than that obtained by dilution plating during first 15 days of storage, but only was significantly different at day 1. Furthermore, an overestimation of viable CPA-2 populations above of 0.30 $\log_{10}$ cells/cm^2 was observed by qPCR when compared with PMA-qPCR.

5.3.2. Study of distribution and persistence of *P. agglomerans* CPA-2 in postharvest applications

The results of sampling conducted on the pre-treatment zone of packing line are shown in Table 1. The samples from all sampling points before treatment did not show the presence of strain CPA-2. Immediately after treatment, CPA-2 was detected (>10^2 CFU per Rodac plate) and confirmed by PCR in the bin dumper (1) and pre-selection roller (2). The process of washing with water on these surfaces was not sufficient to completely remove CPA-2; however, levels of CPA-2 decreased to less than 10 CFU per Rodac plate. After the sampling on day 1, CPA-2 was not detected. Finally, no CPA-2 was detected in both, the air and floor at any time.

The presence of *P. agglomerans* CPA-2 in the treatment zone of packing line (Table 2) was detected after treatment at all surfaces sampled. At sampling day 1, in the spray nozzles (3) and plastic curtain (4) CPA-2 was not detected. However, more than 10 CFU per Rodac of CPA-2 was detected in the brushes (5), even after sampling day 2. After sampling day 3, only 50 % of Rodac plates presented more than 1 colony with phenotypic characteristics similar to *P. agglomerans*, but neither colony was identified as CPA-2. Consequently, the persistence of CPA-2 is less than 1 day in the spray nozzles and plastic curtain and less than 3 days in the brushes. In the air sampling near of this zone, CPA-2 was detectable (>10^2 CFU per Petri plate), but only during treatment application (day 0). Finally, the samples from the floor did not show the presence of strain CPA-2 at any time.

The results corresponding to sampling of the post-treatment zone of packing line are shown in Table 3. Before treatment, the presence of more than 1 colony with phenotypic characteristics similar to *P. agglomerans* was detected, in the distribution belts (8), but neither of them was molecularly confirmed as CPA-2. In the waxing brushes (6), surfaces of drying tunnel (7) and packing table (9), CPA-2 was detected immediately after treatment. The process of washing with water was not sufficient to remove completely CPA-2 in the different surfaces. After the sampling on day 1, CPA-2 was not detected at any sampling surface. The sample from the floor did not show the presence of CPA-2 at any sampling time. Finally, in the air, CPA-2 was detected (>10 CFU per Petri plate), only at sampling day 0. Therefore, the persistence of BCA in post-treatment zone of packing line was less than 1 day, because the CPA-2 strain was not detected after this time.

Table 1. Total number of colonies isolated from pre-treatment zone of packing line with phenotypic characteristics similar to *P. agglomerans* that were identified by conventional PCR to be CPA-2.

Sample	Sampling time (days)	Rodac plate[a]	CFU/Rodac	CPA-2 confirmed by PCR (%)[b]
Bin dumper (1)	Before treatment	0/2	-	-
	After treatment	2/2	$>10^2$	100
	After washing	2/2	>10	100
	1	0/2	-	-
Pre-selection roller (2)	Before treatment	0/2	-	-
	After treatment	2/2	$>10^2$	100
	After washing	2/2	>10	100
	1	0/2	-	-
Air	0	0/2	-	-
	1	0/2	-	-
Floor	0	0/2	-	-
	1	0/2	-	-

[a] Number of plates that showed presence of colonies with phenotypic characteristics similar to *P. agglomerans* in relation to the total number of plates.

[b] Percentage of colonies confirmed to be the CPA-2 strain by conventional PCR. The number of colonies selected was at most 25 colonies per plate, but at least 40 % of all colonies per plate were analysed by conventional PCR.

The total number of colonies isolates from storage chambers with phenotypic characteristics similar than *P. agglomerans* and identified as CPA-2 strain by conventional PCR is shown in Table 4. On different storage chamber surfaces (at 20 °C and 4 °C), such as floors and walls, *P. agglomerans* CPA-2 was not detectable any sampling time. In plastic boxes maintained at both 20 °C and 4 °C, the CPA-2 strain was detected from the first samplings: the initial concentration was more than 10 CFU per Rodac plate at both temperatures. However, at sampling day 1 at 20 °C, meanwhile, the presence of CPA-2 was not detected; however, in plastic boxes maintained at 4 °C, 25 % of samples presented more than 1 colony per Rodac petri, and 100 % were identified as the CPA-2 strain (day 1). Finally, at sampling day 2, CPA-2 was not detected. Consequently, the persistence of CPA-2 is less than 2 days in plastic boxes maintained at 4 °C. Finally, *P. agglomerans* CPA-2 was not detectable in the air of storage chamber at 20 °C. However, in the storage chamber at 4 °C, the CPA-2 was detected and confirmed by conventional PCR only at sampling day 0.

Table 2. Total number of colonies isolated from treatment zone of packing line with phenotypic characteristics similar to *P. agglomerans* that were identified by conventional PCR to be CPA-2.

Sample	Sampling time (days)	Rodac plate[a]	CFU/Rodac	CPA-2 confirmed by PCR (%)[b]
Spray nozzles (3)	Before treatment	0/2	-	-
	After treatment	2/2	$>10^2$	100
	After washing	2/2	>10	100
	1	0/2	-	-
Plastic curtain (4)	Before treatment	0/2	-	-
	After treatment	2/2	$>10^2$	100
	After washing	2/2	>10	100
	1	0/2	-	-
Brushes (5)	Before treatment	0/2	-	-
	After treatment	2/2	$>10^2$	100
	After washing	2/2	$>10^2$	100
	1	1/2	>10	100
	2	1/2	>10	100
	3	1/2	>1	0
Air	0	2/2	$>10^2$	100
	1	0/2	-	-
Floor	0	0/2	-	-
	1	0/2	-	-

[a] Number of plates that showed presence of colonies with phenotypic characteristics similar to *P. agglomerans* in relation to the total number of plates.

[b] Percentage of colonies confirmed to be the CPA-2 strain by conventional PCR. The number of colonies selected was at most 25 colonies per plate, but at least 40 % of all colonies per plate were analysed by conventional PCR.

Results of sampling conducted on the gloves and clothes worn by workers are shown in Table 5. On the gloves, the persistence of the BCA was less than 1 day, because the CPA-2 strain was not detected after this time. In addition, on the working clothes, the CPA-2 was isolated and confirmed at day 0 in all samples. At sampling day 1, no colonies with morphology similar to CPA-2 were found. Consequently, the persistence of the CPA-2 strain on working clothes was less than 1 day.

Table 3. Total number of colonies isolated from post-treatment zone of packing line with phenotypics characteristic similar to *P. agglomerans* that were identified by conventional PCR to be CPA-2.

Sample	Sampling time (days)	Rodac plate [a]	CFU/Rodac	CPA-2 confirmed by PCR (%) [b]
Waxing brushes (6)	Before treatment	0/2	-	-
	After treatment	2/2	$>10^2$	100
	After washing	2/2	>10	100
	1	0/2	-	-
Drying tunnel (7)	Before treatment	0/2	-	-
	After treatment	2/2	$>10^2$	100
	After washing	2/2	>10	100
	1	0/2	-	-
Distribution belts (8)	Before treatment	1/2	>1	0
	After treatment	2/2	$>10^2$	100
	After washing	2/2	>10	100
	1	0/2	-	-
Packing table (9)	Before treatment	0/2	-	-
	After treatment	2/2	>10	100
	After washing	2/2	>10	100
	1	0/2	-	-
Air	0	0/2	>10	100
	1	0/2	-	-
Floor	0	2/2	-	-
	1	0/2	-	-

[a] Number of plates that showed presence of colonies with phenotypic characteristics similar to *P. agglomerans* in relation to the total number of plates.

[b] Percentage of colonies confirmed to be the CPA-2 strain by conventional PCR. The number of colonies selected was at most 25 colonies per plate, but at least 40 % of all colonies per plate were analysed by conventional PCR.

5.4. DISCUSSION

The application system of many BCAs is similar to chemical fungicides and is based on inundative treatment, i.e. either spraying or drenching at high rates. Regardless of the manner of application, BCAs need to be monitored to evaluate their population dynamics, which can be influenced by many factors including time and method of application, ability of BCA to colonize the environment, survival during unfavourable periods, tolerance to climatic change and chemical treatments (Sanzani *et al.* 2014).

In order to see the effect of BCAs in the application environment for registration purposes and knowledge in general, specific analysis methods are necessary to study the traceability of BCAs in the application environment and its survival in surface of fruit. Dilution plating is the quantification method most commonly used to monitor BCAs

(Cañamás *et al.* 2008a,b; De Cal *et al.* 2009), but this method is generally not suitable, because it does not enable the identification of specific strains (Schena *et al.*, 2000). However, genotypic markers are preferable because they are more stable and their expression does not depend on the type of culture media used for analysis. Further, knowledge of nucleotide sequence of these SCAR fragments may form the basis for designing primers for PCR analysis. Currently, some researchers in the area of biocontrol applied to postharvest diseases are beginning to incorporate molecular methods, as qPCR to detect specific BCAs applied in postharvest (Schena *et al.*, 2003; Massart *et al.*, 2005; Spotts *et al.*, 2009). However, one limitation of qPCR is its inability to differentiate between DNA from live and dead cells and consequently could overestimate of potentially viable population. In the last years, DNA-intercalating dye PMA has been used successfully together with qPCR reaction to discriminate and count both live and dead cells in microbiological samples (Nocker and Camper, 2009).

Table 4. Total number of colonies isolated from storage chambers with phenotypic characteristics similar to *P. agglomerans* that were identified by conventional PCR to be CPA-2.

Sample	Storage chamber	Sampling time (days)	Rodac plate[a]	CFU/Rodac	CPA-2 confirmed by PCR (%)[b]
Floor and walls	20 °C	0	0/8	-	-
		1	0/8	-	-
	4 °C	0	0/8	-	-
		1	0/8	-	-
Plastic Boxes	20 °C	0	4/4	>10	100
		1	0/4	-	-
	4 °C	0	4/4	>10	100
		1	1/4	>1	100
		2	0/4	-	-
Air	20 °C	0	0/3	-	-
		1	0/3	-	-
	4 °C	0	1/3	>1	100
		1	0/3	-	-

[a] Number of plates that showed presence of colonies with phenotypic characteristics similar to *P. agglomerans* in relation to the total number of plates.

[b] Percentage of colonies confirmed to be the CPA-2 strain by conventional PCR. The number of colonies selected was at most 25 colonies per plate, but at least 40 % of all colonies per plate were analysed by conventional PCR.

Table 5. Total number of colonies isolated from from handlers' clothes with phenotypics characteristic similar to *P. agglomerans* that were identified by conventional PCR to be CPA-2.

Sample	Sampling time (days)	Rodac plate[a]	CFU/Rodac	CPA-2 confirmed by PCR (%)[b]
Gloves	0	4/4	>10	100
	1	0/4	-	-
Working clothes	0	1/6	>1	100
	1	0/6	-	-

[a] Number of plates that showed presence of colonies with phenotypic characteristics similar to *P. agglomerans* in relation to the total number of plates.

[b] Percentage of colonies confirmed to be the CPA-2 strain by conventional PCR. The number of colonies selected was at most 25 colonies per plate, but at least 40 % of all colonies per plate were analysed by conventional PCR.

In the present work, we have monitored the population of *P. agglomerans* CPA-2 on oranges under two different storage conditions, at 20 and 4 ºC by PMA-qPCR and the results were compared with dilution plating and qPCR. Our results showed that, quantification by PMA-qPCR of CPA-2 populations on oranges stored at both temperatures (20 and 4 °C) were not significantly different to those obtained by qPCR and dilution plating at the initial time of the assay (day 0), confirming the presence of mainly viable and culturable cells at this sampling time. However, the differences between PMA-qPCR and qPCR method increased significantly a long time-course of the assay. Therefore, the bacterial populations were overestimated by qPCR, showing the unreliability of the method for the quantification of viable bacteria when they can be mixed with large numbers of dead cells. Studies conducted by Pujol *et al.* (2006) demonstrated the inability of qPCR method to differentiate between live and dead cells, as its main drawback, when they observed that the population levels of *P. fluorescens* EPS62e NaL estimated by qPCR was approximately 1700 times higher than those CFU counts obtained by dilution plating from leaves of apple trees. Similar results were obtained by Soto-Muñoz *et al.* (2014b) with *P. agglomerans* on apples. Furthermore, population levels quantified by PMA-qPCR were not significantly different compared with populations quantified by dilution plating in fruits stored at 20 °C. In a study conducted by Kramer *et al.* (2009) also observed a good correlation between PMA-qPCR and dilution plating during the quantification of live and dead probiotic bacteria in lyophilized product. Moreover, this study revealed a significant difference between PMA-qPCR and dilution plating in fruits stored during 1 day at 4 °C. A possible explanation for the difference observed between both methods, might be that a portion of CPA-2 cells could be able to remain in the VBNC state, as a strategy of survival and adaptation to conditions of fruit storage and, they could be quantified by the PMA-qPCR method, but not by dilution plating. In fact, some studies indicated that low

temperatures contribute to induction of VBCN in bacteria (Colwell, 2000). Particularly, Pawlowski *et al.* (2011) found that *Yersinia pestis* can induce itself VBCN state, after 46 days of incubation in water at 4 ºC. Moreover, they demonstrated that this bacterium is able to remain with an intact cell membrane that is capable of preventing propidium iodide dye entry. Propidium iodide dye, which like PMA, is selective in penetrating only into dead bacterial cells with compromised membrane integrity but not into live cells with intact cell membranes (Nocker *et al.* 2006). It may be assumed that part of VBNC subpopulations maintained its integrity preventing the PMA diffusion. In addition, in agreement with Nocker *et al.* (2006, 2007) our results showed that PMA treatment efficiently suppressed the amplification of DNA from dead cells.

When bacteria are introduced into a new environment, they are exposed to environmental changes (i.e. low temperature) to which the cells dynamically adapt by genetic mechanisms. Bacteria, with the ability to utilize constitutive and inducible enzyme synthesis, can accommodate to growth-limiting nutrients and adjust or reroute metabolic pathways to avoid metabolic and/or structural disruption caused by specific nutrient limitations. Furthermore, they are able to coordinate their rates of synthesis to maintain their cellular structure and function (Colwell, 2000).

Exposure to microbial cells can occur during handling of a biocontrol product, or even after application. In order to determine the level of risk associated with use of a microorganism, and to manage this risk, it is important to determine the distribution and persistence of a BCA during its handling. Here, we have monitored the persistence of CPA-2 after postharvest application on oranges; the results showed that persistence of CPA-2 was less than 3 and 1 day in surfaces of packing line and working clothes. The CPA-2 presence was not detectable in the environmental and storage chambers surfaces. Our results demonstrate that *P. agglomerans* CPA-2 has a low establishment, survival and dispersal in its application environment. The survival and biochemical activity of BCA may be influenced by the tolerance of the antagonist to the environmental stresses such as dry conditions, direct UV irradiation, high or low temperatures, and low nutrient availability (Nunes, 2012). The relative importance from each of these factors depends on why and where a particular product is used. In application in postharvest (which were our case), the low nutrient availability is probably the most important factor that contributed the low persistence of BCA. The results of this study could answer questions regarding assessment impact of formulation, the risk related to their distribution, because any non-target effects on the environment and/or non-target organisms were observed. Also, these could be used to generate valuable data for the future registration of Pantovital® (BioDurcal S.L. Durcal, Spain) in Europe.

5.5. CONCLUSIONS

In conclusion, PMA-qPCR method is a potential tool to quickly and specifically monitor the viable population of CPA-2. This method gave valuable information on viable population behavior, as well as entry into VBNC state at short-time under cold storage conditions and the presence of the presence of residual DNA from dead cells. On the other hand, the persistence of *P. agglomerans* CPA-2 on different sampling areas after postharvest application was low; due that CPA-2 cannot grown and survive on surfaces sampled. In general, these advances help to understanding the persistence of antagonist when is applied under postharvest conditions and will lead to an optimization of time and mode of application, with beneficial effects on the level of protection that can be achieved, without disregarding the possible risk for the environment.

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DISCUSIÓN GENERAL

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El uso de microorganismos como agentes de control biológico ACBs es una alternativa muy estudiada en los últimos 25 años (Droby *et al.* 2009; Liu *et al.* 2013), siendo uno de los objetivos principales de gran número de investigaciones en todo el mundo (Teixidó *et al.* 2011). En este sentido, el grupo de Postcosecha del IRTA y la Universitat de Lleida ha aislado y estudiado extensamente la bacteria *P. agglomerans* CPA-2 por ser un ACB potencial capaz de controlar las principales podredumbres de postcosecha causadas por los hongos *P. expansum* y *P. digitatum* en manzana y naranja, respectivamente.

En Europa, uno de los factores que dificultan la implementación de un producto de control biológico es la falta de una reglamentación específica que reconozca plenamente las diferencias fundamentales de un ACB respecto a los productos fitosanitarios de síntesis. Los principios para la evaluación y autorización de los productos fitosanitarios (químicos y biológicos) se incluyen en el Reglamento (UE) Nº 546/2011 de la Comisión de 10 de junio de 2011. Estos principios marcan las pautas a seguir para el desarrollo de un producto biológico. Dentro de las pautas establecidas indican que es necesario efectuar una evaluación del destino y del comportamiento del ACB en el medio ambiente teniendo en cuenta el potencial de persistencia y multiplicación de los microorganismos en todos los compartimentos ambientales. Así mismo, el uso de métodos de identificación, detección y cuantificación de los componentes viables e inviables tanto en la formulación como en los residuos presentes en el interior y en la superficie de cultivos tratados.

Los métodos microbiológicos han sido comúnmente utilizados para el seguimiento de ACBs. Sin embrago, estos métodos tienen la desventaja de consumir bastante tiempo para la obtención de resultados, y quizás lo más importante es que pueden subestimar los niveles poblacionales de un ABC cuando éste entra en estado VBCN. En cambio, los

métodos moleculares basados en la PCR, tienen la ventaja de ser altamente específicos, rápidos y muy sensibles. Recientemente, el uso de la qPCR ha surgido como una metodología robusta y ampliamente utilizada en la detección y cuantificación de ACBs (Sanzani *et al.* 2014). El inconveniente de la qPCR, aparte del gran esfuerzo que requiere para su desarrollo, es que en ciertas condiciones, no discrimina las células vivas de las muertas. El uso de agentes intercaladores de ADN, tales como el PMA en combinación con la qPCR permite detectar selectivamente el ADN de las células viables, es por ello que es una alternativa muy prometedora para superar la limitante de la qPCR por sí sola.

Por lo anterior, la presente tesis doctoral tuvo como objetivo contribuir al desarrollo de la qPCR en combinación con el PMA para la detección y cuantificación del antagonista *P. agglomerans* CPA-2 durante su formulación y en la superficie de la fruta. Así como evaluar la dispersión y persistencia del ACB en su entorno de aplicación.

1. DESARROLLO Y OPTIMIZACIÓN DE LAS TÉCNICAS MOLECULARES

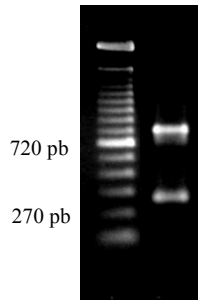
1.1. Desarrollo de la técnica de qPCR

El desarrollo de la qPCR se basa en el reconocimiento de un fragmento del genoma del ACB que sea diferencial al genoma de otras cepas. Para ello, es necesario conocer, si no todo, alguna parte del genoma de nuestra cepa. Generalmente, las estrategias que se han utilizado para conocer la secuencia de un ACB han sido mediante el uso de secuencias disponibles en bases de datos (Braun-Kiewnick *et al.* 2012; Spotts *et al.* 2009), o bien usando los marcadores SCARs (Larena y Melgarejo, 2009; Massart *et al.* 2005; Pujol *et al.* 2006; Schena *et al.* 2002). Esta última estrategia proporciona secuencias diana específicas para el organismo objetivo, por tanto es la más atractiva de utilizar, pero requiere de desarrollo previo de los marcadores SCARs.

El ACB *P. agglomerans* CPA-2 ya cuenta con unos marcadores SCARs denominados PAGA1 y PAGB1 desarrollados previamente por Nunes *et al.* (2008). Por tanto, en la presente tesis estos SCARs han sido el punto de partida para el diseño de cebadores a utilizar en el desarrollo de la qPCR *(Capítulo I)*.

Los SCARs; PAGA1 y PAGB1 generan dos productos de amplificación: uno de 720 pb que es específico de *P. agglomerans* CPA-2 y otro de 270 pb que podría ser un producto diferencial a nivel de especie. Estos productos fueron purificados y enviados al servicio Ez-Seq de Macrogen (Amsterdam, Holanda) para su secuenciación. Una vez conocidas las secuencias se identificó un fragmento diferencial de 143 pb (Figura 1) mediante el alineamiento de ambas secuencias. A partir este fragmento se diseñaron los cebadores SP_2-*forward* (SP_2-F) y SP_2-*reverse* (SP_2-R) con su correspondiente sonda *Taqman*-MGB *(Capítulo I)*, que sirvieron posteriormente para la cuantificación de diferentes

formulaciones de CPA-2 *(Capítulo III)* y para evaluar su dinámica poblacional en la superficie de fruta (*Capítulo IV y Capítulo V*).



TGAGAATCTATCAGCAATAAACAAGCTTAAAGCGCAGGGA
AGCCGGTCAGAAAAGGAAAG??CCAGGACGGCGCGGGCCG
AGCGTCCATGGATGGCTTAAGCGCCTTTCCGATCTGACCG
GTTTCCCTGAGCCGGCTCGATCTCT

Figura 1. Productos de amplificación generados con los cebadores PAGA1 y PAGB1 con ADN de CPA-2 (*P. agglomerans)* y la secuencia de 143 pb diferencial de CPA-2.

La qPCR fue validada verificando la especificidad de los cebadores SP_2-F/SP_2-R y la sonda *Taqman*-MGB, y verificando también la amplificación, optimización, eficiencia y robustez de la reacción (Raymaekers *et al.* 2009).

La **especificidad** de los cebadores SP_2-F/SP_2-R y la sonda *Taqman*-MGB fue demostrada de dos maneras: *(i) In silico* mediante análisis de BLAST *(Basic Local Alignment Search Tool*) explorando las secuencias de ADN disponibles en la base de datos *NCBI GenBank* (www.ncbi.nlm.nih.gov/genbank/) para excluir la presencia de emparejamiento de los cebadores y la sonda con el genoma de algún otro microorganismo. Durante el análisis de BLAST encontramos únicamente la secuencia completa del genoma de *P. agglomerans* IGI disponible en la base de datos. A la fecha, se han introducido en la base de datos otras cuatro secuencias correspondientes a las cepas*: P. agglomerans* DAPP-PG734; 299R, Eh318, y Tx10. *(ii)* Mediante la comparación del producto de amplificación del ADN de *P. agglomerans* CPA-2 con 17 cepas de *P. agglomerans*, 4 de *Pantoea* spp. y otras 21 cepas bacterianas de otros géneros que fueron aisladas de frutas y hortalizas, observándose señal de amplificación sólo para CPA-2.

Es evidente que hay interés en conocer y posteriormente incorporar en la base de datos la secuencia de más microorganismos, pero aún existe poca información referente a los genomas completos de la mayoría de los ACBs. Por tanto, la manera más aceptable de comprobar la especificidad de los cebadores y la sondas es evaluándolos con el mayor número posible de microorganismos, para asegurar que sólo se genera producto de amplificación con el microorganismo diana.

Otro paso que se realizó en esta tesis para la validación de la técnica de la qPCR fue la **verificación de la amplificación** de los cebadores SP_2-F/SP_2-R. Por un lado, los cebadores se analizaron por PCR convencional, obteniéndose un único amplicón de aproximadamente 110 pb (Figura 2). Por otro lado, la reacción en qPCR se verificó determinando la curva de fusión de los cebadores utilizando SYBR Green como detector, confirmándose la presencia de un único pico de fusión a una temperatura de 85 °C, así como la ausencia de dímeros (Figura 3).

Además, las **condiciones de reacción** fueron optimizadas: las concentraciones de 300 nM y 200 nM de sonda *Taqman*-MGB fueron las que emitieron mayor señal de fluorescencia. La concentración de los cebadores coincide con la utilizada en algunos estudios (Pujol *et al.* 2006; Spotts *et al.* 2009), aunque también fueron inferiores a las utilizadas por otros autores (Larena y Melgarejo, 2009; Massart *et al.* 2005). Por tanto, queda patente la necesidad de optimizar la concentración de los cebadores para cada ADN objeto de estudio, dado que puede variar según el tipo del detector, el tamaño del cebador o las condiciones de la reacción (Tagu y Moussard, 2006).

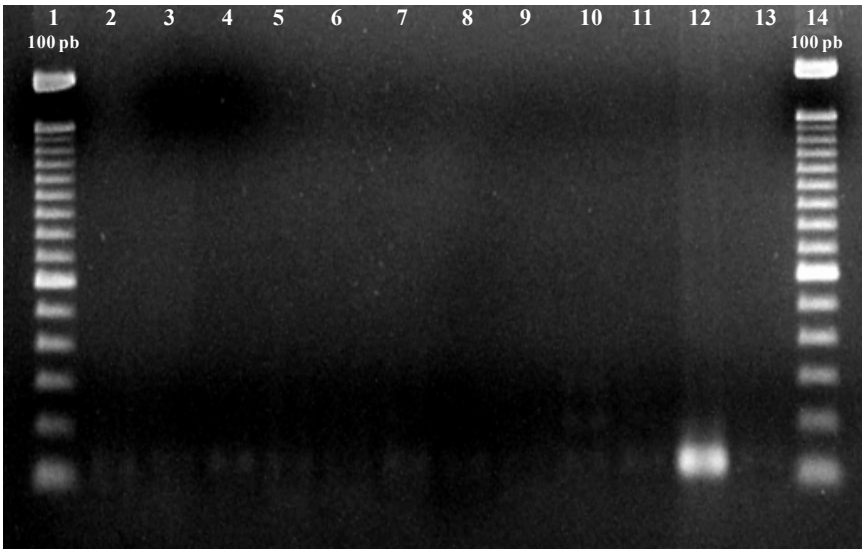


Figura 2. Productos de amplificación generados con los cebadores SP_2-F/SP_2-R con ADN de diferentes cepas. Las columnas 1 y 14 corresponden al marcador de peso molecular 100 bp. Las columnas 2: CCET 850 (*P. agglomerans*), 3: CECT 4842 (*P. agglomerans* 4: CPA-5 (*P. syringae*), 5: EL8 (*Pantoea* spp.), 6: RG4 (*Rahnella aquatilis*), 7: CPA-3 (*P. ananatis*), 8: 128-M (*Pantoea* spp.), 9: PN5 (*Flavimonas oryzihabitans*), 10: M247 (*Pantoea* spp.), 11: CPA-7 (*P. graminis*), 12: CPA-2 (*P. agglomerans*) 13: Control negativo (amplificación con agua).

Para concluir con la validación de la qPCR, se construyeron tres curvas de calibración para determinar la concentración de CPA-2 en cultivo puro (*Capítulo III*) y en la superficie de manzana *(Capítulo I)* y naranja (*Capítulo II, IV y V)* previamente tratados con este ACB. Los valores de las pendientes resultantes fueron de −3.24, −3.15 y −3.08, respectivamente. La curva de calibración que presentó mayor eficiencia de reacción fue con células de CPA-2 procedentes de cultivo puro (103 %), seguida de células recuperadas de manzana (107 %) y naranja (109 %).

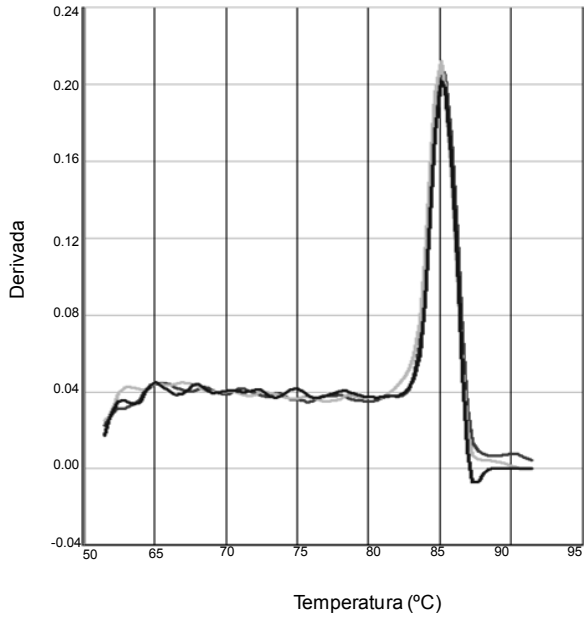


Figura 3. Gráfico de desnaturalización derivado de la fusión del producto obtenido por qPCR usando los cebadores SP_2-F/SP_2-R al amplificar ADN de *P. agglomerans* CPA-2 (detección con SYBR Green).

La pendiente está relacionada con la eficiencia de la PCR, si la pendiente es de -3.32 la eficiencia de la reacción es del 100 %. *Applied Biosystems* (2010) en su manual técnico de qPCR, indica que una eficiencia aceptable para un experimento de qPCR debe presentarse entre 90 y 110 %, lo que confirma que todas las **pendientes y eficiencias** obtenidas para cuantificación de *P. agglomerans* entran en el rango aceptable para la validación de la qPCR. Las variables que pueden afectar la eficiencia de la qPCR pueden ser la presencia de inhibidores de la PCR (como pudo haber sido en nuestro caso) la degradación y concentración del ADN y la longitud del amplicón, entre otras (Raymaekers *et al.* 2009).

Además, la técnica de qPCR aquí desarrollada mostró una alta **robustez**, ya que cada curva de calibración presentó al menos 4 rangos de concentraciones, que oscilaron de 10^3 a 10^8 células de CPA-2 por PCR con valores promedios de *Cq* entre 22 y 38. Valores similares de *Cq* fueron reportados por Massart *et al.* (2005) para la detección y seguimiento del ACB *C. oleophila* (cepa O) utilizando sonda *Taqman*-MGB.

Finalmente, la fiabilidad del método de qPCR se evaluó determinando la dinámica poblacional de *P. agglomerans* CPA-2 en la superficie de manzanas tratadas en postcosecha y conservadas a 20 y 0 °C. Los resultados obtenidos se compararon con el método microbiológico de recuento en placa (*Capítulo I).*

A tiempos cortos de almacenamiento los resultados fueron similares entre ambos métodos. Una tendencia similar fue observada por Spotts *et al.* (2009) al cuantificar *C. infirmominiatum* sobre frutos tratados con este ACB. Sin embargo, en los últimos muestreos de la dinámica poblacional de *P. agglomerans* CPA-2 la cuantificación por qPCR fue altamente superior a la obtenida con el método de recuento en placa. Estudios realizados por Pujol *et al.* (2006) confirman la diferencia en los niveles poblacionales de *P. fluorescens* EPS62e NaL estimados por el método de qPCR y el de recuento en placa en hojas de manzano. Dichos autores atribuyen esta diferencia a la presencia de células en un estado VBCN, así como a la presencia de ADN libre no degradado proveniente de células muertas. Mientras que en nuestro estudio, podemos concluir que las células de CPA-2 que se aplicaron sobre los frutos se encuentran en excelentes condiciones, sin ningún tipo de estrés. Por tanto, no se detectó la presencia de células en estado VBCN al momento de la aplicación. En cambio, en los últimos muestreos de la dinámica poblacional, qPCR tuvo la limitación de no distinguir entre el ADN procedente de células viables, muertas y aquellas que se encuentran en estado VBCN.

Los ACBs difieren fundamentalmente de los fungicidas químicos en que para poder ser efectivos debe mantenerse, crecer y proliferar en el ecosistema donde son aplicados. Esto significa que las cepas antagonistas deben permanecer viables para ser activas frente a los patógenos diana durante los períodos favorables a la infección (Usall *et al.* 2013). Por tanto, para estudiar el comportamiento de un ACB es crucial distinguir las células viables de las no viables.

1.2. Desarrollo de la metodología del PMA en combinación con qPCR

Una estrategia que se siguió en la presente tesis para superar la limitación de la qPCR fue introducir, antes de la cuantificación por qPCR, un pre-tratamiento de la muestra con el PMA.

Para la implementación de esta técnica fue necesario establecer las condiciones óptimas de concentración de PMA, tiempo de incubación y foto-exposición para células de *P. agglomerans* CPA-2 viables, no-viables y una mezcla de las mismas. Estas condiciones fueron evaluadas tanto en cultivo puro como en una matriz de naranja (*Capítulo II)*. Asimismo, se evaluó el efecto citotóxico de este intercalador sobre CPA-2.

Las condiciones establecidas para el pre-tratamiento con PMA fueron: 30 µM de PMA, 20 min de incubación en oscuridad y 30 min de foto-exposición con luz LED azul (LED del acrónimo en inglés, *light-emitting diode*). Además, 50 mM de PMA mostró un efecto citotóxico sobre las células de *P. agglomerans* CPA-2.

En las muestras con naranja se realizó un paso de filtración para evitar interferencias de la matriz de naranja. Es posible que esto evitara diferencias al aplicar el PMA a cultivo puro de CPA-2 y en una matriz de naranja. Las matrices ambientales y alimenticias suelen ser muy complejas, el contenido de compuestos orgánicos e inorgánicos pueden interferir con la fotoactivación (Fittipaldi *et al.* 2012). Además de otros factores como la turbidez, sal y el pH, o una alta concentración de células muertas. Crespo-Sempere *et al.* (2013) observaron que la matriz de tomate interfería en la cuantificación de esporas viables de *Alternaria* spp.

Al comparar la técnica PMA-qPCR con el método de qPCR y el de recuento en placa en células viables de CPA-2 suspendidas en una matriz de naranja no se observó diferencias significativas entre los tres métodos, a pesar que estas células habían sido sometidas a un proceso de liofilización. De acuerdo con Nocker *et al.* (2006) el PMA tiene la capacidad de penetrar selectivamente en las células con daño en membrana, y mediante una reacción de fotolisis se une al ADN, suprimiendo la amplificación del ADN de las células muertas. En este estudio, se demostró la capacidad del PMA en suprimir la amplificación del ADN procedentes de células no viables de *P. agglomerans* CPA-2.

2. APLICACIÓN DE LAS TÉCNICAS MOLECULARES EN LA CUANTIFICACIÓN DE *Pantoea agglomerans* CPA-2

Una vez desarrollada la técnica de PMA-qPCR, se aplicó a la formulación de *P. agglomerans* CPA-2 *(Capítulo III)* y para evaluar su dinámica poblacional en la superficie de naranjas tratadas en condiciones de pre- *(Capítulo IV)* y postcosecha *(Capítulo V)*.

2.1. Cuantificación de *P. agglomerans* CPA-2 durante su formulación

El método de cuantificación PMA-qPCR *(Capítulo II)* fue comparado con la técnica de qPCR (*Capítulo I*) y de recuento en placa para determinar la supervivencia de *P. agglomernas* CPA-2 en tres métodos de deshidratación comúnmente utilizados para la formulación de un ACB; atomización, liofilización y lecho fluido. Para la rehidratación de los formulados se utilizaron dos diferentes medios: leche descremada en polvo (NFSM) y agua *(Capítulo III)*. Los resultados mostraron que la recuperación de células viables de CPA-2 con agua fue igual a la obtenida con leche descremada, en los tres formulados. Además, las células de CPA-2 liofilizadas mostraron mayor supervivencia en comparación a los resultados obtenidos en lecho fluido y atomización.

Los métodos de cuantificación mostraron una tendencia diferente en cada método de deshidratación:

(i) En las células deshidratadas por **liofilización** no se observó diferencia significativa entre los tres métodos. Estos resultados sustentan los encontrados durante la optimización de la técnica del PMA-qPCR *(Capítulo II)*. Kramer *et al.* (2009) encontraron una tendencia similar al determinar la viabilidad de células liofilizadas de *Lactobacillus acidophilus* y *Bifidobacterium animalis* spp. por el método de PMA-qPCR y qPCR y recuento en placa. Por tanto, los tres métodos de

cuantificación podrían ser utilizados para determinar la viabilidad en este tipo de formulación.

(ii) En el proceso de lecho fluido, la qPCR sobreestimó la viabilidad de las células formuladas, mientras que el PMA-qPCR y el recuento en placa no mostraron diferencias. Por tanto, la pérdida de cultivabilidad de la célula se puede atribuir a la presencia de daños en la membrana ocasionadas en este proceso de secado.

(iii) En el método de deshidratación por atomización, los tres métodos de cuantificación fueron estadísticamente diferentes. Al igual que en el lecho fluido, la qPCR mostró una sobreestimación de las células viables. Entre el método del PMA-qPCR y el recuento en placa se observó una diferencia de 2.17 $\log_{10}$ UFC o células/g de materia seca. Las diferencias entre los tres métodos puede deberse a daños en la membrana debido a las altas temperaturas que se utilizan durante este proceso de secado (Fernández-Sandoval *et al.* 2012), y a otros daños que no afecten a la permeabilidad de la membrana, como son los daños estructurales en los ribosomas, en el ADN, la oxidación y carbonilación de proteínas, entre otros. Estos daños pueden ser reparados por la célula y no afectar a su viabilidad, o bien pueden inducir a un estado VBCN o la muerte celular (Peighambardoust *et al.* 2011; Shafiei *et al.* 2014).

Este último resultado, nos llevó a plantearnos la siguiente cuestión: estas células ¿conservarán su capacidad antagonista para controlar al patógeno? Para dar respuestas a esta pregunta, nos plateamos evaluar la viabilidad y eficacia de las células atomizadas de *P. agglomerans* CPA-2 en naranjas previamente heridas e inoculadas con *P. digitatum*, y ajustando la concentración del tratamiento basándonos en el método de recuento en placa y en el del PMA-qPCR. Las células formuladas fueron rehidratadas con agua y leche descremada. Por un lado, los resultados mostraron que la leche descremada actuó mejor como rehidratante que el agua al presentar mayor control sobre el crecimiento del hongo a los 3 días de incubación. Sin embargo, este control no se mantuvo a los 4 días de incubación. Por lo que no se observaron diferencias entre rehidratar con leche descremada y agua. Por otro lado, la dosis de aplicación ajustada con la técnica de PMA-qPCR fue 1.70 $\log_{10}$ UFC/g de materia seca inferior que la dosis ajustada con el método de recuento en placa. A pesar de esta diferencia ambas dosis mostraron el mismo control sobre *P. digitatum* a los 3 y 4 días de incubación. Por tanto, esto nos llevó a deducir que la técnica de PMA-qPCR permitió detectar y cuantificar aquellas células que entran en estado VBCN, las cuales no son detectadas por el método microbiológico de recuento en placa.

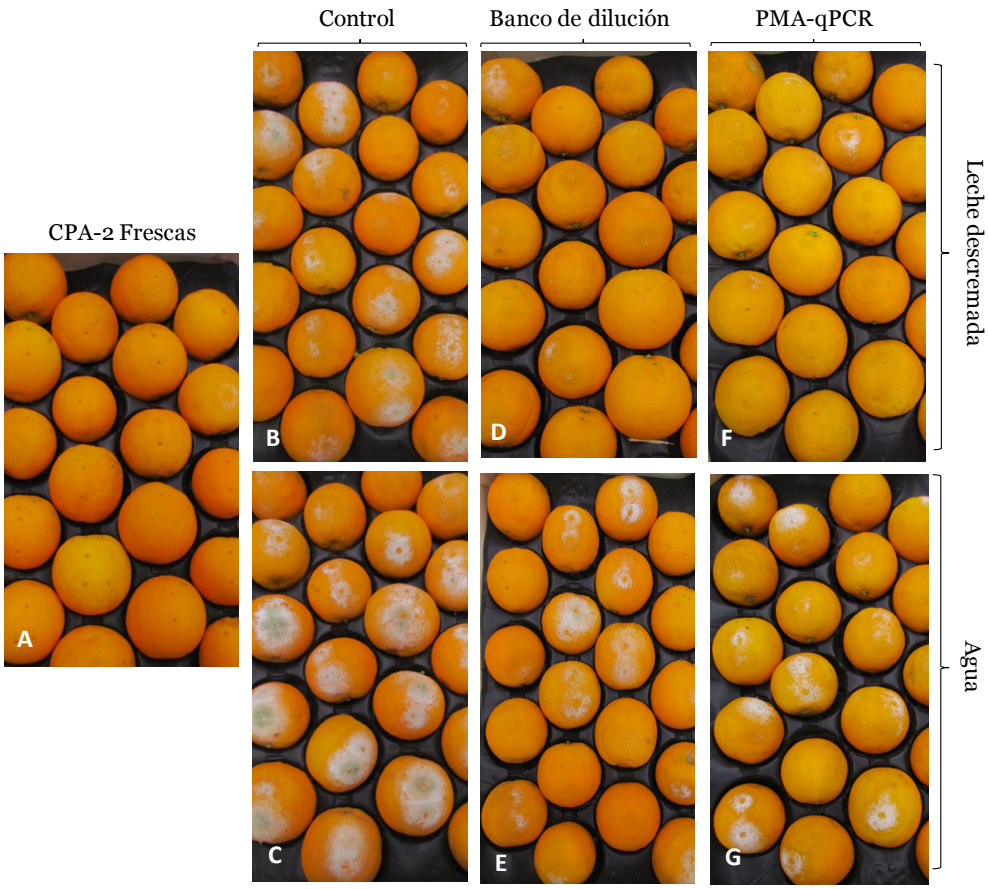


Figura 4. Efectividad del atomizado de *P. agglomerans* CPA-2 a 10^8 UFC/mL rehidratados con leche descrecremada (D,F) y/o agua (E,F), ajustando la dosis mediante la técnica de cuantificación de banco de dilución (D,E) y/o qPCR-PMA (F,G) contra el patógeno *P. digitatum* (10^3 conidias/mL) en naranja *Valencia Late* después de 4 días a 20 °C, comparado con células frescas de CPA-2 (A), patógeno + leche descremada (B) y patógeno+agua (C).

Por tanto, considerando que el modo de acción de CPA-2 es la competencia por el espacio o los nutrientes (Poppe *et al.* 2003), podemos deducir que la técnica de PMA-qPCR permitió detectar y cuantificar tanto las células viables cultivables como las que entran en estado VBCN, que no son detectadas por el método microbiológico de recuento en placa, ya que fueron capaces de colonizar las heridas de naranja y prevenir el desarrollo de *P. digitatum*, aunque en menor medida que las células frescas.

2.2. Cuantificación de *P. agglomerans* CPA-2 en la superficie de las naranjas tratadas en pre- y postcosecha

Al igual que los productos químicos, los productos basados en microorganismos pueden aplicarse tanto en precosecha, para prevenir la infección en el campo y evitar que la fruta llegue a la central infectada, como en postcosecha para prevenir la infección a través de las heridas y disminuir infecciones ya establecidas, evitando así su desarrollo y dispersión durante el período de postcosecha (Usall *et al.* 2013).

El producto de *P. agglomerans* CPA-2 formulado por liofilización fue aplicado sobre naranjas *Valencia Late* en condiciones de precosecha *(Capítulo IV)* y en postcosecha *(Capítulo V)*. Se eligió el método de liofilización para todos estos ensayos porque es el método de formulación escogido para el producto comercial. En ambos estudios, se evaluó la dinámica poblacional de CPA-2 recuperada de la superficie de los frutos mediante el método de recuento en placa, PMA-qPCR y qPCR. Inmediatamente después del tratamiento (tiempo 0), se observaron niveles poblacionales de CPA-2 más elevados en los frutos tratados en precosecha que en los frutos tratados en postcosecha, con una diferencia aproximada de 0.70 $\log_{10}$ UFC o células/cm^2. La diferencia entre ambos ensayos radica principalmente en la metodología y en las dosis de aplicación. En este caso, la diferencia entre ambos ensayos puede deberse a un efecto de la metodología de aplicación, más que a la diferencia en concentraciones de aplicación, debido a que en el ensayo en precosecha la dosis de aplicación (2x10^8 UFC/mL) fue inferior a la que se aplicó en postcosecha (2x10^9 UFC/mL).

En general, los niveles poblacionales de CPA-2 se mantuvieron más estables a lo largo del tiempo en condiciones de postcosecha que en precosecha, con niveles poblacionales por encima de los 3.65 $\log_{10}$ UFC/cm^2 después de 30 días de almacenamiento a 4 °C. Tanto los frutos del interior como del exterior de los árboles tratados en campo mostraron niveles poblacionales inferiores a los obtenidos en postcosecha, alcanzando niveles poblacionales de alrededor 1.45 $\log_{10}$ UFC/cm^2 después de los 16 días de la aplicación del tratamiento. Estos resultados parecen lógicos, ya que la cepa CPA-2 es un microorganismo adaptado a condiciones de frigoconservación y además las condiciones en cámara son mucho más estables que las condiciones en campo, donde existen una serie de estreses ambientales como son la radiación solar, la desecación, las altas temperaturas, entre otros, que pueden provocar lesiones subletales en la célula y afectar la viabilidad del ACB (Kinkel *et al.* 2000).

La persistencia de CPA-2 puede estar influenciada por la tolerancia de antagonista a las tensiones ambientales, tales como condiciones secas, la irradiación UV directa, altas temperaturas, baja disponibilidad de nutrientes, y los cambios climáticos rápidos

(Nunes, 2012). Cualquiera de estos factores de estrés pueden provocar lesiones subletales a una parte de la población (Kinkel *et al.* 2000). Aunque, en aplicaciones en entornos foliares (que eran nuestro caso), la radiación solar y especialmente porción ultravioleta del espectro, es probablemente el factor más importante que afecta a la persistencia de un BCA (Filho *et al.* 2001). Por lo tanto, nuestros resultados demuestran que *P. agglomerans* CPA-2 no presentaría un riesgo inaceptable para el medio ambiente debido a su baja supervivencia y dispersión en el medio ambiente.

Las condiciones ambientales a las que se enfrenta el microorganismo en campo son más drásticas que las condiciones que se puede encontrar en una cámara de almacenamiento. Además, se conoce que el tratamiento de precosecha es menos efectivo que los de postcosecha en el control de infecciones de herida, ya que sólo parte del tratamiento aplicado permanecerá en los frutos para proteger las heridas que se produzcan más tarde. No obstante, existen diversos estudios que indican que los tratamientos en precosecha pueden ser efectivos para reducir determinadas enfermedades de herida, como es la causada por *P. digitatum* en naranja (Cañamas *et al.* 2008).

En cuanto a los métodos de cuantificación evaluados en ambos estudios en naranjas, la qPCR mostró resultados similares que el método de PMA-qPCR y recuento en placa a tiempos iníciales. Sin embargo, a lo largo del tiempo las poblaciones cuantificadas por la qPCR se mantuvieron constantes, tal como se mostró en el estudio con manzana *(Capítulo I)*. Independientemente de las condiciones de aplicación, o del tipo de fruto, la qPCR presentó la misma limitación de sobreestimar la población de CPA-2, porque con este método se cuantifican viables y no viables. Por tanto, este método puede ser utilizado para conocer de manera rápida y específica la concentración de CPA-2 inmediatamente después de la aplicación del tratamiento, pero no es un método que permita dar seguimiento y evaluar el comportamiento de CPA-2 en la superficie de la fruta a lo largo del tiempo, porque cuando empiezan a morirse las células el método deja de ser fiable.

Por otra parte, los niveles poblacionales de *P. agglomerans* CPA-2 cuantificados por PMA-qPCR fueron estadísticamente diferentes a los obtenidos por recuento en placa, después de 3 días de la aplicación del tratamiento en campo. En cambio, en postcosecha no se observó diferencias significativas entre el PMA-qPCR y el método de recuento en placa. Las condiciones ambientales del campo (temperatura, luz, humedad, radiación, entre otras) pueden inducir al microorganismo a un estado VBCN, como estrategia de supervivencia (Oliver, 2010), y no ser detectado por el método de recuento en placa, pero sí por el método de PMA-qPCR. De acuerdo con Nocker y Camper (2009), el PMA tiene la capacidad de penetrar solamente en células bacterianas muertas con la integridad de la

membrana comprometida pero no en células vivas con membranas celulares intactas. En condiciones de almacenamiento, seguramente la lisis celular es lo que produce la pérdida de viabilidad de CPA-2. Y posiblemente por ello no se observó diferencia entre el método de PMA-qPCR y el de recuento en placa en los frutos tratados en postcosecha.

Los valores de población obtenidos mediante PMA-qPCR fueron significativamente inferiores a los obtenidos por qPCR, lo cual demuestra la capacidad del PMA para diferenciar entre las células vivas de CPA-2 y las que mueren debido a las condiciones ambientales de campo, o durante el almacenamiento de la fruta.

3. EVALUACIÓN DE LA DISPERSIÓN Y PERSISTENCIA DE *Pantoea agglomerans* CPA-2 DURANTE SU APLICACIÓN EN PRE- Y POSTCOSECHA

En la actualidad, la normativa europea vigente ((UE) Nº 546/2011) exige una evaluación rigurosa de los riesgos antes de registrar un fungicida biológico como producto fitosanitario, y antes de su introducción en el mercado. Esta evaluación debe incluir información sobre la capacidad del ACB para colonizar, persistir y propagarse, así como las posibles rutas de dispersión en condiciones habituales de uso. Sin embargo, el reglamento no define los métodos para establecer los niveles basales de un microorganismo autóctono cuando se va a utilizar como ACB.

La combinación de técnicas microbiológicas clásicas con herramientas moleculares podría ser una estrategia adecuada para el seguimiento durante y después de la aplicación del ACB para conocer su eficacia como método ecológicamente seguro en el control de enfermedades. Nunes *et al.* (2008), emplearon el recuento de UFC/mL y la PCR convencional (usando marcadores SCARs) para la cuantificación y seguimiento de *P. agglomerans* CPA-2 en naranja en tratamientos postcosecha. En esta tesis se han utilizado estos dos métodos para determinar la distribución y persistencia del ACB cuando es aplicado en precosecha *(Capítulo VI)* y postcosecha de naranjas *(Capítulo V)* y/o manzana *(Capítulo I)*.

3.1. Estudio en precosecha (naranjas)

La dispersión durante la aplicación del tratamiento de *P. agglomerans* CPA-2 en campo y su capacidad de colonizar y persistir en el ambiente, así como en otros sitios no diana como, hojas, suelo/tierra y hierba contribuyen, en cierta medida, a comprender el comportamiento y el impacto medioambiental de CPA-2 *(Capítulo IV)*.

Uno de los objetivos de esta tesis fue evaluar la dispersión y persistencia de *P. agglomerans* CPA-2 durante su aplicación en un campo comercial sobre árboles de naranja *Valencia Late* (Figura 5).

Figura 5. Aplicación del tratamiento de *P. agglomerans* CPA-2 en campo

Los principales resultados mostraron que el tratamiento de CPA-2 **no se dispersa** a distancias superiores de 2.5 ± 0.5 m. La parte exterior de los árboles no-tratados situados alrededor de los árboles tratados mostraron cantidades del ACB menores al 1 % en los papeles hidrosensibles. En cuanto a la **persistencia** de CPA-2 en el ambiente, ésta fue inferior a 21 días, mientras que en hojas y frutos de los árboles no tratados y en malas hierbas localizadas por debajo de los árboles no tratados fue inferior a 16 días. En el suelo CPA-2 no sobrevivió después del tratamiento; en cambio, en las hojas de los árboles tratados fue capaz de persistir 16 días. En las malas hierbas localizadas por debajo de los árboles tratados se observó un número importante de colonias con características fenotípicas similares a *P. agglomerans*, sin embargo, al hacer la confirmación por PCR convencional no correspondían a CPA-2. Este microorganismo fenotípicamente parecido fue aislado e identificado mediante secuenciación del ARNr 16s como *P. ananatis*.

P. agglomerans y *P. ananatis* están estrechamente relacionados y son difíciles de identificar en placa, debido a la gran similitud fenotípica, la falta de características distintivas y una taxonomía algo confusa (Deletoile *et al.* 2009). En el caso de evaluarse

la persistencia con el método microbiológico, los resultados no hubieran sido reales. Por tanto, podemos afirmar que el uso de los SCARs es una herramienta útil para identificar células de *P. agglomerans* CPA-2 aplicada en campo y distinguirla de otras cepas con características fenotípicas similares.

Los microorganismos antagonistas liberados en campo están expuestos a una variedad de factores ambientales que pueden influir en su establecimiento y persistencia, como son la desecación, lluvia, radiación UV, baja disponibilidad de nutrientes, así como los cambios drásticos de temperatura (Nunes, 2012). Cualquiera de estos factores pueden ser estresantes para el microorganismo y pueden provocar lesiones subletales a una parte de la población (Kinkel *et al.* 2000). Concretamente, en aplicaciones en entornos foliares, la radiación solar, y especialmente la porción ultravioleta del espectro, es probablemente el factor más importante que afecta a la persistencia de un ACB (Filho *et al.* 2001). Así mismo, la composición físico-química del suelo podría estar relacionada con la persistencia del microorganismo. Cabe señalar que la cepa de *P. agglomerans* CPA-2 utilizada es estos estudios ha sido adaptada a condiciones de estrés, incluso es crecida en un medio que la hace más resistente a este tipo de condiciones (Teixidó *et al.* 2006; Cañamas *et al.* 2008).

3.2. Estudio en postcosecha (naranjas y manzanas)

La principal estrategia utilizada en todo el mundo para el control de podredumbres de frutas en postcosecha son los tratamientos en central frutícola. Los productos a base de ACBs en postcosecha pueden aplicarse en ducha (sistema conocido popularmente como *drencher*) o por aspersión directamente en la línea de confección. Ambos sistemas requieren una manipulación adicional de la fruta antes de su traslado a las cámaras de almacenamiento.

Con el fin de determinar el comportamiento del ACB en su entorno de aplicación hemos evaluado la distribución y persistencia de *P. agglomerans* CPA-2 en postcosecha de manzana aplicada en drencher *(Capítulo I)* y en línea de confección sobre naranja *(Capítulo V)* para el control de *P. expansum* y *P. digitatum*, respectivamente. En ambos estudios se realizaron diferentes muestreos: en la zona de aplicación de tratamiento, en la de conservación de fruta y en los EPIs utilizados por los operarios (guantes, botas, ropa de trabajo).

Los resultados mostraron que el sistema de aplicación está estrechamente relacionado con la persistencia de CPA-2. En general, las principales diferencias que se encontraron en la zona de tratamiento entre ambos estudios fueron:

(i) En el sistema de aplicación en *drencher* se encontraron concentraciones más elevadas de *P. agglomerans* CPA-2, que en la línea de confección de cítricos en los diferentes puntos de muestreo. La diferencia en volumen de tratamiento que se necesita en cada sistema de aplicación puede estar contribuyendo en esta diferencia. Es decir, para tratar 10 cajas con 20 Kg de fruta en *drencher* se necesitaron al menos 100 L de tratamiento, mientras que en la línea de confección 5 L del tratamiento es suficiente para tratar la misma cantidad de fruta.

(ii) La persistencia estuvo directamente relacionada con la concentración de ACB; en manzanas la dosis recomendada es 2 x 10^7 UFC/mL y en naranjas 2 x 10^9 UFC/mL. A mayor concentración de *P. agglomerans* CPA-2, mayor persistencia, como ya parece lógico. En las superficies del *drencher* la persistencia de CPA-2 fue inferior a 1 día, mientras que en la línea de confección fue de 3 días.

(iii) En *drencher* se detectó la presencia de CPA-2 en suelo, no así en línea de tratamiento. Después de la aplicación, en la misma línea de confección la fruta tratada pasa por un túnel de secado, en cambio, en *drencher* la fruta bañada se transporta a cámaras de almacenamiento sin un secado previo, con lo que el exceso de agua de tratamiento cae directamente al suelo.

(iv) En la línea de confección hubo una dispersión del tratamiento en el ambiente por las microgotas que se producen en las boquillas por donde se aplica el tratamiento, mientras que en *drencher* no se detectó presencia alguna de CPA-2 en el aire en ningún tiempo evaluado.

En la zona de conservación no se detectó la presencia de CPA-2 en ninguna de las superficies de cámaras de conservación. En cambio, las cajas de plástico usadas para almacenar la fruta en frío presentaron mayor persistencia, siendo de 5 días cuando la fruta se trató en *drencher* y de 3 días en la línea de confección.

Los EPIs presentaron una mayor exposición al ACB cuando se realizaron los tratamientos en *drencher* que en línea de confección, siendo la máxima persistencia de 2 días en los equipos de protección utilizados por los operarios. Además, la persistencia de *P. agglomerans* CPA-2 encontrada en la ropa de los operarios que realizaron el tratamiento en precosecha (inferior a 7 días) *(Capítulo III)* fue mayor que en la ropa de los operarios que hicieron el tratamiento en postcosecha (inferior a 2 días) *(Capítulo IV y V)*. La exposición del operario aplicador en precosecha es superior que en postcosecha, en que la línea y el *drencher* funcionan automáticamente y de forma bastante independiente del operario.

Nuestros resultados demuestran que *P. agglomerans* CPA-2 tiene una limitada dispersión y una baja persistencia, cuando es aplicada en postcoscha, ya sea en *drencher*

ó en la línea de confección. La baja persistencia de CPA-2 podría atribuirse a diversos factores, como la composición de la superficie, los nutrientes disponibles, entre otros. A diferencia de precosecha, la mayoría de las superficies muestreadas en postcosecha son zonas inertes para el ACB, carentes de nutrientes y de difusión restringida. Ante esta circunstancia, el microorganismo puede entrar en una situación de estrés provocándole daños celulares que se traducen en la pérdida de permeabilidad de la membrana, inhibición en la síntesis de proteínas, ribosomas, ADN y muerte celular (Fernández, 2008).

En resumen, el desarrollo de las técnicas moleculares qPCR y PMA-qPCR, son una herramienta útil para estudiar el comportamiento de *P. agglomerans* CPA-2 con la ventaja de obtener resultados más rápidos que el método de recuento en placa. Debido a la limitación de la qPCR, de no distinguir células vivas de muertas, sólo permite determinar la población de CPA-2 en la superficie de la fruta inmediatamente después de su aplicación, ya sea en condiciones de precosecha o postcosecha y puede usarse como control de la aplicación.

En cambio, la técnica PMA-qPCR permite evaluar el comportamiento de CPA-2 a través del tiempo en la superficie de la fruta y durante su formulación mediante liofilización, lecho fluido y atomización gracias a su capacidad de cuantificar de manera específica las células vivas de *P. agglomerans* CPA-2.

Un factor limitante para el registro de un ACB es la falta de información sobre el riesgo ambiental generado por la introducción de microorganismos vivos en el medio ambiente. Para ello, es necesario el conocimiento científico y los estudios que nos permitan evaluar el comportamiento en el medio ambiente del ACB en diversas condiciones de aplicación. Los estudios de distribución y persistencia nos proporcionaron una mejor comprensión del destino ambiental y la persistencia de CPA-2 cuando es aplicado en campo y en dos sistemas de aplicación en postcosecha. Todos estos datos, nos servirán como herramientas útiles para la optimización de las formulaciones, tiempo y modo de aplicación, con efectos beneficiosos sobre el nivel de protección que se pueden conseguir, sin descuidar el posible riesgo para el medio ambiente. Estos estudios han proporcionado datos valiosos para el futuro registro de *P. agglomerans* CPA-2 en Europa.

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CONCLUSIONES

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1. *Desarrollo del método de qPCR*

- ✓ Se identificó un fragmento diferencial del genoma de *P. agglomerans* CPA-2 que permitió el diseño de unos cebadores (SP_2-F/SP_2-R) y una sonda *Taqman*-MGB específicos para CPA-2, cuyas eficiencias y límites de detección resultaron aceptables para la validación de la qPCR.

- ✓ La técnica de qPCR desarrollada resultó ser una herramienta útil para la cuantificación de *P. agglomerans* CPA-2 inmediatamente después de su aplicación sobre fruta en condiciones pre- y postcosecha. Sin embargo, no permite evaluar la dinámica poblacional de CPA-2 sobre la fruta a medio y largo plazo.

2. *Desarrollo de la técnica de PMA-qPCR*

- ✓ Un pre-tratamiento de la muestra con 30 μM de PMA, 20 min de incubación en oscuridad y 30 min de foto-exposición en combinación con la qPCR permitió la cuantificación selectiva de células viables de *P. agglomerans* CPA-2.

- ✓ El PMA a una concentración de 50 μM presenta un efecto citotóxico sobre las células de CPA-2.

3. *Aplicación del PMA-qPCR durante la formulación de P. agglomerans CPA-2*

- ✓ La utilización de leche descremada en polvo como rehidratante no mejoró la recuperación de las células de *P. agglomerans* CPA–2 en comparación con el agua en los tres tipos de formulados estudiados (liofilización, atomización y lecho fluido).

- ✓ Las células de *P. agglomerans* CPA-2 liofilizadas fueron las que presentaron mayor supervivencia y no se observaron diferencias significativas entre los tres métodos de cuantificación evaluados (recuento en placa, qPCR y PMA-qPCR).

- ✓ En los formulados secados por lecho fluido, la técnica de PMA-qPCR no mostró diferencias significativas con respecto al método de recuento en placa. En cambio, una diferencia significativa de 2.20 $\log_{10}$ UFC o células/g de materia seca se observó entre ambos métodos de cuantificación en los atomizados de *P. agglomerans* CPA-2.

- ✓ En los tres productos formulados, la técnica de qPCR sobreestimó la población de células vivas de *P. agglomerans* CPA-2.

- ✓ En el ensayo de efectividad en naranja *Valencia Late,* los tratamientos con el atomizado de *P. agglomerans* CPA-2 ajustando concentración/dosis mediante los métodos de cuantificación de PMA-qPCR y el microbiológico de recuento en placa, mostraron el mismo control sobre *P. digitatum* a los 3 y 4 días de incubación, disminuyendo la severidad e incidencia del patógeno, aunque en menor medida que las células frescas, demostrando que en cierta manera las células viables no cultivables tienen cierta efectividad en el control de los patógenos.

4. *Evaluación de las dinámicas poblacionales de* P. agglomerans *CPA-2 en pre- y postcosecha de naranja mediante método microbiológicos y moleculares.*

- ✓ Los niveles poblacionales de *P. agglomerans* CPA-2 fueron más elevados en los frutos tratados en precosecha que en los frutos tratados en postcosecha, inmediatamente después de la aplicación del tratamiento. Sin embargo, los niveles poblacionales de CPA-2 se mantuvieron más estables a lo largo del tiempo en condiciones de postcosecha que en precosecha.

- ✓ Los niveles poblacionales de *P. agglomerans* CPA-2 cuantificados por el método de PMA-qPCR fueron significativamente superiores que los obtenidos por el método de recuento en placa después de 3 días de la aplicación del tratamiento en precosecha. En cambio, no se observaron diferencias en los niveles poblacionales de CPA-2 entre ambos métodos de cuantificación en los frutos tratados en postcosecha y almacenados a 20 y 4 °C.

- ✓ Los niveles poblacionales de *P. agglomerans* CPA-2 cuantificados por la técnica de qPCR mostraron una alta correlación con el método de PMA-qPCR y el de recuento en placa inmediatamente después del tratamiento tanto en precosecha como en postcosecha; sin embargo, qPCR sobreestimó la población viable de CPA-2 a lo largo de los ensayos.

5. *Dispersión y persistencia del tratamiento de* P. agglomerans *CPA-2*

- ✓ En aplicaciones en precosecha, el tratamiento de *P. agglomerans* CPA-2 no se dispersó a distancias superiores de 2.5± 0.5 m en campo. La persistencia de CPA-2 en el aire fue inferior a 21 días, mientras que en hojas y frutos de los árboles no tratados fue inferior a 16 días. En el suelo no se detectó la presencia del antagonista después del tratamiento. En las hojas y los frutos de los árboles tratados, y en las malas hierbas CPA-2 persistió durante 16 días después del tratamiento.

- ✓ En aplicaciones postcosecha, la máxima persistencia de *P. agglomerans* CPA-2 fue de 1 y 3 días en las superficies del *drencher* y en la línea de confección, respectivamente. En las cámaras de almacenamiento no se detectó la presencia de CPA-2, mientras que en las cajas de plástico utilizadas para conservar la fruta su persistencia máxima fue de 5 días. Finalmente, en los EPIs utilizados por los operarios en los tratamientos precosecha y postcosecha, la persistencia de CPA-2 fue inferior a 2 y 7 días, respectivamente.

CONSIDERACIONES FINALES

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Al finalizar esta extensa tesis, conviene que me detenga a reflexionar y trate de responder algunas preguntas:

¿Cuáles han sido las aportaciones de esta tesis en el desarrollo del ACB P. agglomerans CPA-2 para su futura aplicación?

Las aportaciones más importantes de esta tesis han sido el desarrollo de técnicas moleculares, como la qPCR sola y en combinación con el PMA (PMA-qPCR) que permitirán la cuantificación rápida y específica de células viables del ACB CPA-2 en diferentes etapas de su desarrollo: producción, formulación, estudios de la dinámica poblacional en fruta y en el medio ambiente.

La qPCR cuantifica tanto el ADN proveniente de células viables como de las no viables; por tanto, esta técnica podría utilizarse cuando la totalidad de la población es viable; es decir, en los momentos iniciales de su aplicación para constatar la concentración inicial de CPA-2. Además, como una herramienta de control de calidad durante su formulación mediante liofilización.

La técnica combinada del PMA-qPCR desarrollada en esta tesis nos permite detectar y cuantificar tanto las células cultivables como las VBCN de CPA-2, siendo ésta una de las aportaciones más novedosas de esta tesis. Esta técnica podría tener múltiples aplicaciones prácticas en el desarrollo a escala industrial de este ACB, por ejemplo: *(i)* determinar la viabilidad de células de CPA-2 en la fase de producción, *(ii)* determinar la concentración de células viables formuladas por liofilización, atomización y lecho fluido, *(iii)* evaluar el seguimiento poblacional de CPA-2 a medio y largo plazo después de su aplicación tanto en precosecha como en postcosecha, como control de calidad del tratamiento, y *(iv)* como una herramienta para determinar la tolerancia del ACB cuando es sometido a diferentes tipos de estrés.

Cabe señalar que esta es la primera investigación en la que se desarrolla y aplica la técnica del PMA-qPCR para el estudio de un ACB en pre- y postcosecha. Por lo tanto, se trata de un estudio pionero en el ámbito del control biológico.

Por otro lado, esta tesis también contribuye a la información necesaria para el registro y futura comercialización de CPA-2 ya que ha quedado demostrada su baja persistencia y limitada dispersión después de su aplicación tanto en precosecha como en postcosecha. Estos estudios suponen concluir el programa de desarrollo de este ACB. Además, se han establecido los protocolos para los estudios de dispersión y persistencia los cuales podrían ser el modelo a seguir para otros ACBs, debido a que se carecen de protocolos establecidos para este fin.

Y finalmente, *¿Qué tareas deben llevarse a cabo en un futuro próximo?*

Una vez conocida la persistencia de CPA-2, el siguiente paso sería comprobar el impacto ambiental de este agente de biocontrol, mediante técnicas moleculares como es la electroforesis en gel desnaturalizante en gradiente (DGGE) ó realizando estudios de metagenómica microbiana.

Por último, se podrían realizar estudios de microscopía electrónica de transmisión con las células de CPA-2 para conocer los daños que se producen durante su formulación, lo cual permitiría optimizar su formulación. Sería también interesante el uso de estas técnicas para determinar los daños que sufren las células de los ABCs cuando son expuestos a condiciones de estrés.

En conclusión, con esta tesis se ha dado un paso adelante en el uso de ACBs y se abren nuevas puertas a futuros estudios.